Novel Roles of the Inflammatory Cytokine Oncostatin-M in Breast Cancer Pathogenesis

by

Nathaniel R. West
B.Sc., University of Victoria, 2007

A Dissertation Submitted in Partial Fulfillment
of the Requirements for the Degree of

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SUPERVISORY COMMITTEE

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ABSTRACT

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Despite ongoing advancement in detection and treatment, breast cancer remains a major clinical challenge worldwide. Cancer has traditionally been conceptualized as a 'disease of the genes' by virtue of the mutagenic events necessary for its inception. It is now clear, however, that complex interactions take place between cancer cells and the array of non-cancerous cells and molecules in their immediate surroundings, known generally as the tumour microenvironment. Cancer-microenvironment interactions are increasingly recognized as processes that critically influence the outcome of disease.

Cells of the host immune system are major components of the breast tumour microenvironment. While their presence in tumours is thought to reflect an attempt at disease eradication or containment, cancer cells can exploit the immune system through a variety of means, including the recognition of leukocyte-derived cytokines. As such, intratumoral leukocytes and high cytokine content are frequently associated with aggressive subtypes of breast cancer and poor prognosis. This dissertation explores the influence of one such cytokine, oncostatin-M (OSM), on the behaviour of breast cancer cells. Our results collectively demonstrate that OSM can rapidly and potently induce aggressive features in well-characterized cell models of luminal, well-differentiated breast cancer. These features include suppression of the important biomarker estrogen receptor-α (the key molecular target of endocrine therapy), gain of the breast cancer oncogene S100A7, loss of luminal epithelial differentiation and gain of mesenchymal features, and induction of a phenotype consistent with breast cancer stem cells. Each of these changes can potentially influence treatment responsiveness, the metastatic process, or both.

Along with high levels of intratumoural leukocytes, the OSM-induced features listed above are known to associate with one another in human breast cancer. Tumours that display such characteristics have a poor prognosis and present the greatest challenges for modern breast cancer therapy, both because they are inherently prone to rapid metastasis and because targeted therapies for such tumours are lacking. The etiology of these aggressive disease subsets is largely unknown, and resolution of this issue would represent a major advancement in our understanding of breast cancer. Importantly, we found that expression of OSM and/or its receptor OSMR was reproducibly associated with these features in multiple breast cancer cohorts, largely confirming our experimental results. OSMR, in particular, was associated with poor clinical outcome. OSM signalling may thus provide a novel mechanistic explanation for the development of aggressive forms of breast cancer. If our findings are validated and expanded upon in future studies, OSM signalling could serve as a novel therapeutic target and may be an important consideration in the design and deployment of breast cancer immunotherapies.

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Where to begin? As many graduate students before me have likely done, I will first express my deep gratitude to my supervisor since 2007, Dr. Peter Watson. Far from acting as the tryannical overlord that students stereotypically envision their supervisors to be, Peter has provided a constant source of calm direction and constructive criticism. Interaction with Peter is encouragingly collegial; even in the early days of my graduate work, he was (at least seemingly) happy to hear my ideas and engage in a two-way scientific conversation. As such, I have yet to leave his office feeling stupid. I am deeply grateful for Peter's trust in allowing me to pursue self-directed objectives which, I believe, has allowed me to develop problem solving and planning skills to an extent that I might have not otherwise achieved. A key benefit of Peter's supervision is his infectious passion for both the clinical and basic realms of cancer research. This has unexpectedly allowed me to participate in multiple translational studies. Through him I have also learned to appreciate the oft-overlooked importance and subtleties of biobanking, for which I am grateful. Finally, I am thankful for the little extras that so enrich one's training, such as a new appreciation for the importance of peatiness in scotch and excellent British words like 'nobbled.' Peter is an exceptional supervisor, and I would recommend him as a mentor to any aspiring graduate student.

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DEDICATION

To my grandmother Diane, my aunt Karen, and my childhood surrogate mother Cathy. Your inspirational battles with cancer constantly reinforce my sense of purpose.

CHAPTER 1—Introduction

1.1—Prologue

Breast cancer is the most common female malignancy worldwide, striking one in nine Canadian women during the course of their lives. The global breast cancer incidence in 2008 was nearly 1.4 million, with a death toll of over 450,000.[1] The profound societal impact of breast cancer has rightfully placed it as one of the most actively studied human diseases, and the resulting knowledge has been translated into considerable clinical advances in the past several decades. Nevertheless, there is increasing recognition that the old paradigms of cancer biology, which depend heavily on cell-intrinsic concepts of malignancy, are insufficient to explain the diverse and complex behaviour of breast cancer observed in the laboratory and clinic. Rather than being entities driven only by mutagenesis, breast tumours (and cancers in general) are now widely considered to be products of mutagenic events and the tumour microenvironment; that is, the array of normal tissues, cell types, and associated molecules that surround and interact with malignant cells. The tumour microenvironment represents a dauntingly complex and variable element in the etiology and progression of cancer, and is a frontier that we have only recently begun to explore. If we are to truly understand malignancy and provide optimal care for cancer patients, an adequate knowledge of the contribution of the microenvironment is paramount.

A key component of the tumour microenvironment is the host immune system, including leukocytes and leukocyte-derived cytokines. The immune system has a critical role in preventing and controlling the development of cancer through a process commonly referred to as immune surveillance. Unfortunately, the existence of clinically detectable tumours demonstrates their ability to escape eradication by the immune system. Moreover, tumours appear capable of exploiting immune activity to support their own survival and progression. The core objective of the tumour immunology field is to understand these processes to facilitate the development of therapies that tip the immunological balance in favour of tumour eradication. In the setting of breast cancer, this thesis will explore a novel mechanism of immune exploitation based on the leukocyte-derived cytokine oncostatin-M (OSM).

While this thesis is primarily focused on tumour biology, concepts of cancer immunology are important aspects of the research described herein. In the following sections of this chapter, I have attempted to outline the key concepts required to understand the rationale and content of the

[1] Source: American Cancer Society. *Global Cancer Facts & Figures 2nd Edition.* Atlanta: American Cancer Society, 2011.

studies described in chapters 2 to 4. These sections will include discussions of (a) breast cancer biology and clinical management, (b) estrogen receptor-α (ER) and its role in breast cancer therapy, (c) tumour immunology, (d) cytokine signalling in breast cancer, with particular attention to OSM and its family members, and (e) the breast cancer oncogene S100A7. The aim of this chapter is to relate each of the above topics to one another in a logical way, discuss the known or suspected links between them, and introduce outstanding questions pertinent to the work in this thesis.

1.2—Breast cancer biology and clinical management

Breast cancer has been documented in text since ancient times, described by the Egyptians as early as 3,000 BC.[2] Though some individuals will insist otherwise, our knowledge of and ability to treat breast cancer has improved considerably since then. The past 40 years, in particular, have seen great strides in the forms of chemotherapy and modern approaches to endocrine therapy. This section will focus on the biological basis of breast cancer, followed by current approaches to clinical management.

1.2.1—Breast histology and pre-malignant changes

The parenchyma of the breast consists of a branching ductal tree with its base at the nipple. The ducts are supported by a vascularized fibrous stroma with varying degrees of adipose tissue involvement, and culminate in grape-like clusters of dilated dead-end tubules known as terminal duct lobular units (TDLU (1)). The ducts and TDLUs have a simple structural arrangement of two cell layers, consisting of cuboidal luminal epithelial cells surrounded by a layer of spindle-shaped myoepithelial cells (2). The luminal cells are responsible for milk production during lactation, and the myoepithelial cells contract in response to oxytocin to eject milk during nursing. The myoepithelial cells have an additional crucial role in maintaining the integrity of the ductal network, as they are primarily responsible for the production of the laminin-rich basement membrane that surrounds ductal structures. In the absence of myoepithelial cells and, crucially, laminin-1, luminal epithelial cells fail to form lumina and have reversed polarity (3, 4).

[2] Source: American Cancer Society. *The History of Cancer.* Atlanta: American Cancer Society, 2011.

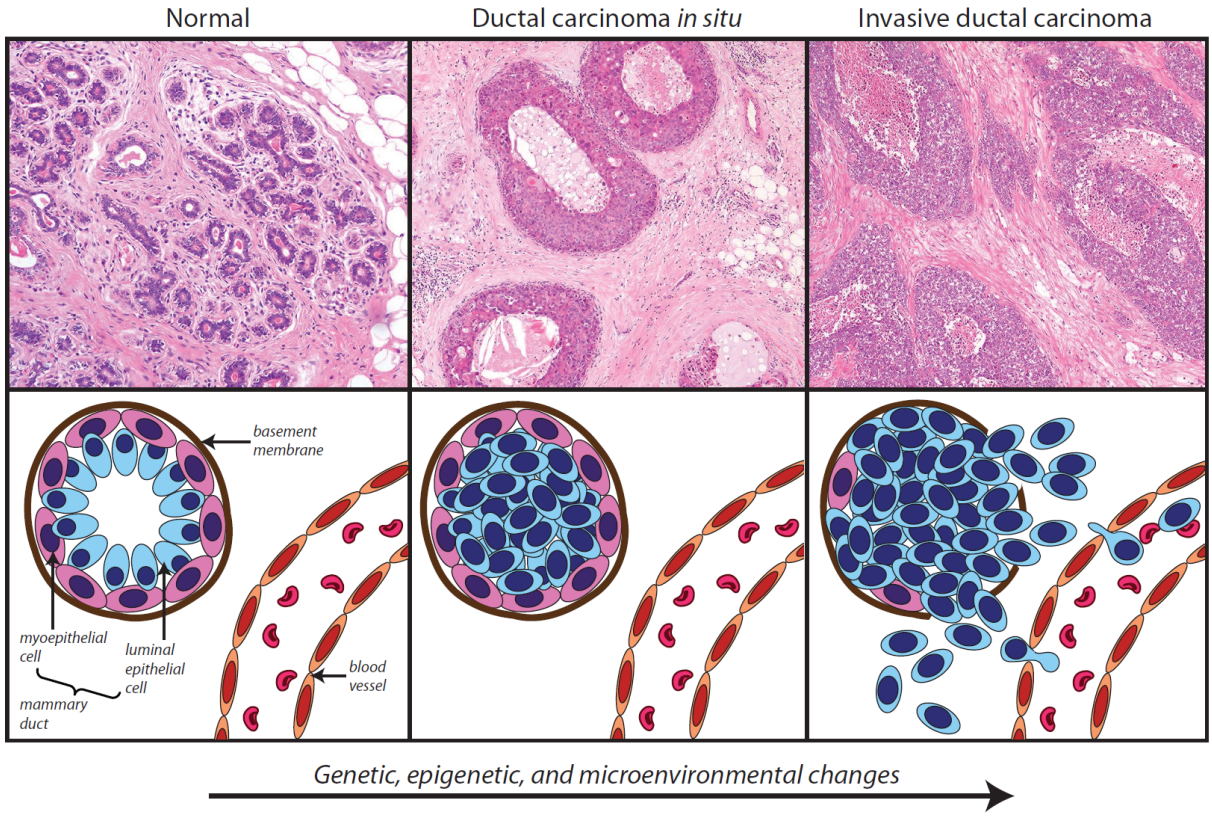

Figure 1. **The progression of breast cancer**. Normal cells within the TDLU mutate, progress through several benign proliferative stages, and may eventually form an *in situ* carcinoma, typically of ductal or lobular histology (the most common form, ductal, is depicted in this example). At this stage, despite rapid proliferation, cancer cells are spatially restricted by the myoepithelial cell layer and the basement membrane. In many *in situ* carcinomas, the integrity of the myoepithelial layer is eventually compromised and cancer cells penetrate beyond the basement membrane to form an invasive carcinoma. At this stage, invading cancer cells have the potential to disseminate via vascular or lymphatic routes and form metastases at distant sites.

The reigning histopathological theory of breast cancer evolution holds that tumours develop through a sequential series of benign pre-invasive stages characterized by varying degrees of cellular proliferation and atypia (Fig. 1). Eventually, advanced benign lesions acquire key mutations that lead to generation of non-invasive *in situ* carcinomas, most commonly ductal carcinoma *in situ* (DCIS). For example, the p53 tumour suppressor is mutated or inactive in 20–30% of DCIS lesions, but not in atypical ductal hyperplasia, the suspected benign precursor of DCIS (1). Nearly all invasive cancers are thought to arise from *in situ* carcinomas, as illustrated by shared chromosomal aberrations and gene expression profiles between DCIS cells and those of adjacent invasive lesions (5-7). Although DCIS is, genetically speaking, a fully fledged carcinoma, DCIS patients have a

remarkably high 10 year survival rate of least 98% following surgical treatment (8). This is attributed to DCIS being spatially restricted by the myoepithelial layer and associated basement membrane, which together prevent the spread of tumour cells into adjacent tissues and systemic circulation. However, DCIS cells can eventually gain the ability to escape the confines of the basement membrane to form a fully malignant invasive tumour. Through unknown mechanisms, this event is frequently associated with loss of functional myoepithelial cells and basement membrane proteins at the stromal-epithelial boundary (2). Due to the lack of evidence for significant or reproducible differences between synchronous pre-invasive and invasive breast cancers that might account for the onset of invasion, it is possible that changes in the microenvironment are required for development of invasive disease (9). This concept was supported in a recent study by Ma *et al* (10), who used laser-capture microdissection to isolate stroma from pre-invasive and invasive ductal carcinomas. Unlike malignant epithelia, the authors noted substantial changes in stromal gene expression associated with gain of invasiveness, particularly with respect to proteases involved in extracellular matrix remodeling. Interestingly, Ma *et al* (10) also noted that stroma from high grade tumours (a histological feature associated with increased risk of the *in situ* to invasive transition (11)) was enriched for genes associated with leukocytes. Indeed, abnormally high levels of leukocytes and stromal activity are commonly observed in DCIS (9). Although these data support a role for the tumour microenvironment, the precise mechanisms by which stromal cells can promote the invasive transition remain uncharacterized.

1.2.2—Invasive breast cancer: histological and molecular diversity

Invasive breast cancer (termed simply breast cancer hereafter) is a highly heterogeneous disease, both clinically and biologically. The most common histological type of breast cancer is ductal carcinoma, which accounts for 50–80% of all cases, followed by lobular carcinoma (5–15%) and a variety of histological 'special' types that collectively account for the remainder (12). These classifications do not refer to a cell of origin, but are rather defined on the basis of the structural and cytological features observed in pathological specimens. Indeed, the vast majority of breast cancers, regardless of histological type, are thought to arise from cells of the TDLU, the most rapidly proliferating cells of the ductal tree (13, 14).

Because the biological underpinnings and clinical impact of the histological subtypes are unclear, they have had a modest impact on clinical practice. As such, most breast cancer researchers and clinicians are more familiar with several key molecular biomarkers related to tumour behaviour

and treatment responsiveness. The three most clinically important breast cancer biomarkers are ER, progesterone receptor (PR), and human epidermal growth factor receptor 2 (Her2). ER is the primary receptor for 17β-estradiol (estrogen) and a key player in both breast tissue development and carcinogenesis. At clinical presentation, roughly 70% of breast cancers are classified as positive for ER expression. Generally speaking, these tumours have a good prognosis, both because they tend to have favourable baseline clinical features (notably, smaller tumour size and low grade (15)) and because they are treatable by endocrine therapy, a class of systemic therapy designed to disrupt estrogen signalling. PR expression is highly dependent on ER and is therefore useful as a marker of ER functionality (16, 17). Her2 is overexpressed in about 15% of breast cancers and is associated with poor prognosis. However, patients with Her2+ tumours benefit significantly from treatment with the Her2-specific monoclonal antibody trastuzumab (Herceptin™ (16, 17)). Expression levels of these biomarkers vary widely among tumours. For example, while in some tumours ER is expressed in every neoplastic cell, breast cancers are classified as clinically ER-positive if as few as 1% of tumour cell nuclei show immunohistochemical reactivity (18).

The rise of transcriptome-profiling technologies in the last 15 years has engendered a distinct field of breast cancer taxonomy based on the assessment of multi-gene expression patterns. Perou *et al* (2000 (19)) provided the first evidence that breast tumours could be subdivided into families based on expression of key sets of genes. Since then, this finding has been validated repeatedly, establishing the existence of at least five 'intrinsic' molecular subtypes of breast cancer: luminal-A, luminal-B, Her2, basal-like, and normal breast-like (20, 21). Luminal tumours (34–66% of breast cancers) are described as having gene expression patterns that resemble those of luminal epithelial cells, including luminal cytokeratins, ER, PR, and other genes associated with ER signalling. These tumours tend to have a good prognosis and favourable clinical features such as low histological grade. Relative to luminal-A tumours, luminal-B tumours are notable largely for higher rates of proliferation and a somewhat poorer prognosis. Tumours of the Her2 subtype (4–10%) express genes consistent with activation of the Her2 pathway or amplification of the Her2 genetic locus. These are typically ER-negative (ER–) and have a poor prognosis. Basal-like tumours (16–37%) are typically negative for ER, PR, and Her2 ('triple negative,' in clinical jargon) and express genes found in mammary myoepithelial cells such as epidermal growth factor receptor (EGFR) and cytokeratins 5 and 14. These tumours also have a poor prognosis, are typically high grade, and frequently harbour dense lymphocytic infiltrates. Notably, the large majority of tumours arising in *BRCA1* mutation carriers have a basal-like phenotype (22). Normal breast-like tumours (≤10%) are poorly defined,

with intermediate clinical outcomes and transcription patterns that appear similar to normal breast stroma. It is possible that this class represents an artifact based on tumour samples that are rich in stroma with limited neoplastic content. Recently, an additional subtype previously grouped with basal-like tumours has been described. This 'claudin-low' subtype comprises poor-prognosis tumours characterized by triple negative status, low expression of genes that encode intercellular junction proteins, and high expression of genes associated with the immune system and stem cells (23). As an example of the degree of phenotypic divergence between these subtypes, Bertucci *et al* (24) assessed the number of differentially expressed genes between luminal-A and basal-like breast cancers and demonstrated a degree of divergence equivalent to that observed between basal-like breast cancer and colorectal cancer. Notably, the expression of ER, PR, Her2, and genes related to these markers are integral components of the gene expression patterns that define the intrinsic molecular subtypes. As such, intrinsic subtype definitions have not proven superior to ER, PR, or Her2 in predicting tumour responses to current therapies, and our knowledge of these subtypes has consequently had little impact on the clinical management of breast cancer.

While our awareness of breast cancer diversity is rapidly expanding, we have yet to explain its etiology. On this issue, two general schools of thought exist (25-27). The stem cell-of-origin model postulates that tumours arise from mutations within the mammary stem cell compartment, based on the premise that stem cells are long-lived residents of breast tissue and can thus accumulate the mutations necessary to generate tumours. In this context, it is envisioned that transformed stem cells (also known as tumour initiating cells) undergo asymmetric division to both maintain the cancer stem cell population and produce relatively differentiated daughters that proliferate to form the bulk of the tumour. Phenotypic diversity is thought to arise based on the specific differentiation pathways taken by the progeny of transformed stem cells. A similar theory suggests that tumours of distinct phenotypes arise due to mutations in specific populations of mammary progenitors (for example, luminal-type tumours forming from mutated luminal progenitor cells). However, our ability to test this hypothesis is currently limited by our ignorance of the normal stem cell hierarchy of the mammary gland (27). The second model, known as the clonal evolution and selection hypothesis, suggests that any cell in the mammary gland can give rise to cancer, provided the acquisition of appropriate genetic changes. In this model, competition among distinct subpopulations of cells leads to outgrowth of favourable variants and the manifestation of diverse tumour phenotypes (26). To date, as the referenced reviews describe (25-27), the origins of breast cancer diversity have been explored from a largely tumour cell-centric viewpoint, with emphasis placed on the genetic,

epigenetic, and transcriptional changes of malignant cells. Although researchers frequently invoke Darwinian selection as a fundamental process in cancer development, the role of the microenvironment in tumour evolution is often neglected. This is important because, as with any biological system, the environment is an important source of selective pressures that influence the evolution of organisms. Indeed, the bewildering array of mutations found in breast cancer (28), few of which occur with reasonable frequency, implies that knowledge gained from a purely genetics-based approach will not be sufficient to fully explain breast cancer biology.

1.2.3—Breast cancer metastasis

At diagnosis, 5% of breast cancer patients have already developed clinically detectable metastatic disease (29). Of the remainder, approximately one third develop distant metastases following treatment, from which point the median survival time is roughly three years (30). The most common sites of breast cancer metastasis (in decreasing order) are the bones, liver, lungs, and brain (31). Metastatic breast cancer is generally incurable using current therapies, and patients are thus treated with life-extending and palliative intent. As such, the vast majority of breast cancer related deaths are due to metastasis. A complex series of processes are required to successfully metastasize, beginning with the transition from an *in situ* lesion to a locally invasive tumour (32-35). Invasion requires both migration and the capacity to penetrate the extracellular matrix. Once invasive cells gain access to vascular and lymphatic vessels, they must undergo intravasation (penetration of vessel walls to enter circulation), survive circulatory transit to a distant site, extravasate (exit from circulation to enter foreign tissue), and survive in the novel environment to form a new tumour. It is widely accepted that dissemination and metastasis occurs with disease progression, as implied in Figure 1. With respect to the specific timing of these processes, however, it is now appreciated that single tumour cells can disseminate during early stages of tumourigenesis, and that metastases may develop in parallel with the primary tumour (36).

Although the processes described above are well established prerequisites of metastasis, clear molecular explanations for them remain elusive. Invasion typically involves dissolution of cell-cell adhesion complexes and rearrangements in cell-matrix interactions, such that single cells can exit the primary tumour and migrate through surrounding tissues. A similar process that occurs during embryogenesis, particularly in events such as gastrulation and neural crest migration, is known as the epithelial-to-mesenchymal transition (EMT (37, 38)). Although the actual relevance of EMT to human cancer progression remains controversial, largely owing to difficulties in detecting this

process in clinical specimens (39), EMT has become a popular topic of cancer research. The spectrum of phenotypic changes that are thought to constitute EMT include loss of epithelial markers such as E-cadherin and other intercellular junction proteins; gain of mesenchymal markers including vimentin, fibronectin, and N-cadherin; nuclear localization of β-catenin and/or expression of key transcription factors such as snail, slug, and twist; and, most importantly, loss of epithelial polarity with concurrent acquisition of mesenchymal morphology and migratory capacity. Importantly, EMT can bestow resistance to anoikis (a form of apoptosis induced by loss of substrate adhesion), which is crucial for dissemination via the circulatory system (38, 40). Mesenchymal changes in cancer cells can be induced through various mechanisms, including cytokine signalling (particularly transforming growth factor beta (TGFβ)), receptor tyrosine kinases, Notch activation, and hypoxia (37). Although the hallmarks of EMT are frequently observed in experimental models, carcinomas may in reality express mesenchymal features to varying degrees. For example, a recent study demonstrated that loss of E-cadherin and expression of N-cadherin, snail and slug are frequently observed in independent cohorts of human breast cancer. However, while snail and slug expression correlated with one another, they were not related to loss of E-cadherin or gain of N-cadherin, nor were E-cadherin and N-cadherin inversely correlated, as would be expected for tumours that had undergone a full EMT (41). This implies that tumours may gain invasiveness by exhibiting only a subset of EMT features. Furthermore, because poor differentiation (i.e., high grade) is regarded by some as a correlate of cancer cell invasiveness, the apparent gain of mesenchymal features in invasive tumours may simply be a result of deregulated epithelial differentiation, rather than the orchestrated expression of a latent embryonic program. As such, cancer cells may be more appropriately described as undergoing an 'EMT-like' process during local invasion and dissemination (40). This is supported by the apparent reversibility of EMT in cancer, whereby metastatic tumour cells regain expression of an epithelial phenotype in a process known as the mesenchymal-to-epithelial transition (MET; (37, 42)). Thus, while EMT-like processes may underlie the invasion and dissemination steps of metastasis, the majority of invasive cancer cells likely exist in a 'metastable' phenotype that incorporates elements of both epithelial and mesenchymal differentiation.

In recent years, the previously distinct fields of EMT and cancer stem cells have become inextricably linked, beginning with a report in 2008 by Mani and colleagues (43). These authors demonstrated that both normal and transformed mammary stem cells express genes consistent with EMT, and that induction of EMT in mammary cells leads to acquisition of stem cell-like features.

Similar results were reported by Morel *et al* three months later (44) and several others have expanded on this concept since then (45). Cancer stem cells (CSCs) are thought to constitute a small proportion of a tumour's bulk (typically less than 1%) and act as the primary agents of tumour growth and dissemination. In this context, the term 'cancer stem cell' does not necessarily imply that tumours arise from transformed stem cells, nor should it be taken to mean that CSCs exhibit all the features of normal stem cells. Rather, CSCs are merely thought to exhibit phenotypes that are similar to stem cells, such as resistance to cytotoxic agents, the ability to undergo asymmetric division, and the expression of genes normally restricted to primitive, undifferentiated cell types. The major observation that led to this concept was that individual leukemic cells have unequal tumour-forming abilities *in vivo*. That is, only a small fraction of malignant cells are capable of generating disease, and these can be enriched using biomarker-based and functional assays (46).

In breast cancer, by far the most common method of identifying putative CSCs is by assaying for a $CD44^{+}CD24^{low/-}$ cell surface phenotype (45, 47, 48). In their original description by Al-Hajj *et al* (48), $CD44^{+}CD24^{low/-}$ cells isolated from human breast tumours were enriched by up to 50-fold for tumourigenicity in immune deficient mice relative to the bulk tumour cell population. Furthermore, sorted populations of these cells gave rise to xenografts that contained the full diversity of cells observed in the original tumours. While the gold standard approach for demonstrating tumour-initiating ability in putative CSCs is low-titer passaging and serial transplantation *in vivo*, an *in vitro* test known as the mammosphere assay has been established as a reliable method for selecting cells with stem cell-like and tumour initiating traits. First used for this purpose in 2003 by Dontu *et al* (49), the mammosphere assay involves culturing single cells in adhesion-free and serum-free conditions with media containing defined growth factors. Normal mammary cells capable of surviving and forming three-dimensional colonies in these conditions are capable of regenerating an entire ductal tree *in vivo* (50). Cancer cells that behave likewise are highly efficient at forming tumours *in vivo* and express markers consistent with stem cells (51). Because of their ability to efficiently spawn new tumours, CSCs are postulated to be the progenitors of metastatic lesions. The potential role of CSCs in breast cancer metastasis is supported by the observation that most disseminated cancer cells in the bone marrow of breast cancer patients possess a $CD44^{+}CD24^{low/-}$ phenotype, whereas these make up only a small fraction of the primary tumour (52). Similarly, high grade tumours and those from poor prognosis molecular subtypes (particularly basal-like and claudin-low tumours) appear to be enriched in stem cell-like features (23, 53, 54). CSCs are also thought to underlie the resistance of tumours to cytotoxic therapies such as

ionizing radiation and chemotherapy. Putative CSCs from breast cancer cell lines (identified by $CD44^{+}CD24^{low/-}$ status or mammosphere-forming capacity) are relatively radiation resistant (55, 56), and anti-tumour immune responses in murine mammary tumours can lead to the selective outgrowth of radio- and chemoresistant CSC-like cells (57). Furthermore, cells with CSC properties are enriched in human breast tumours following chemotherapy treatment (58, 59).

Aside from forced expression of EMT transcription factors such as snail and twist, the actual physiological mechanisms that support the breast cancer stem cell phenotype are unclear. As with EMT, several cytokines have been reported to enhance breast CSC features, including TGFβ, tumour necrosis factor-α (TNF), interleukin (IL)-6, and IL-8 (43, 44, 60-62). In addition, hypoxia can activate expression of the Notch ligand Jagged2 in breast cancer and thereby promote EMT and outgrowth of cancer stem-like cells (63). Thus, limited evidence supports a role for the tumour microenvironment in triggering and/or augmenting stem cell-like features in breast cancer.

1.2.4—Breast cancer treatment

Breast cancer patients are assigned specific therapies based largely on the pathological features of their tumours, such as grade, tumour size, nodal status, and biomarker expression (ER, PR, and Her2). Several pathological terms appear frequently in this thesis and should thus be briefly defined. 'Grade' refers to a semi-quantitative assessment of cellular atypia that is employed as a predictor of prognosis by pathologists. Although several systems for grading breast cancer exist, they generally involve a composite assessment of three parameters: the extent to which normal glandular differentiation is retained by malignant cells, the number of visible mitoses, and the degree of nuclear pleomorphism (variation in nuclear size). Low grade (grade 1) tumours have relatively normal histology and generally good prognosis, while intermediate and high grade (grades 2 and 3) lesions have worse outcomes, with poorer differentiation, higher mitotic rates, and increased nuclear pleomorphism (64). 'Nodal' status refers to the presence (node+) or absence (node–) of metastatic cancer cells in local (generally axillary) lymph nodes. The presence of lymph node metastasis is strongly associated with an increased risk of disease recurrence. Tumour size is generally categorized into one of four 'T' sizes as follows: T1 (≤20 mm), T2 (>20 mm but ≤50 mm), T3 (>50 mm), and T4 (any size with direct tumour extension to the chest wall or skin). Finally, anatomic 'stage' classifies the extent (and thus prognosis) of disease by combining nodal status, tumour size, and the presence or absence of clinically detectable metastasis. Stages range from 0 (*in situ* disease) to IV (breast cancer with distant metastasis (65)).

The primary treatment for breast cancer through the 20th century was mastectomy, which entails the complete surgical removal of the affected breast along with, in some cases, local lymph nodes and underlying pectoral muscle. This approach has largely given way to what is known as breast-conserving surgery (BCS) or 'lumpectomy,' in which only the tumour itself is removed. Axillary lymph nodes are often removed in the context of BCS as well, but this component of the surgical approach has become more restricted over time with the realization that the main benefit of axillary lymph node sampling in the absence of clinically detectable nodal metastasis is to establish nodal status for staging. Thus, removal of the so-called 'sentinel' node is now a common alternative to extensive axillary dissection. The use of radiotherapy in conjunction with BCS is of particular importance, as it reduces 5-year local recurrence rates from 26% (with BCS alone) to 7%, with an associated 5% reduction in 15-year overall mortality (66). As such, BCS with radiation therapy is now frequently used, particularly given the rising numbers of breast cancers detected at early stages due to improved mammographic screening.

Following surgery, occult malignant cells may remain either locally or at distant sites and can trigger disease recurrence up to 20 years following the initial diagnosis. While radiotherapy is administered to help prevent local recurrence, systemic therapy is typically administered to control metastatic foci of disease and reduce the rates of distant recurrence. In this context, systemic therapies are administered following surgery and are defined as a form of 'adjuvant' therapy. Systemic therapy given prior to surgery is known as 'neoadjuvant' therapy. This typically occurs in cases of locally advanced cancer, with the aim of reducing tumour burden to augment surgical resection. Current systemic therapies for breast cancer mainly include polychemotherapy (combinations of two or more cytotoxic drugs with distinct mechanisms of action), endocrine therapy (agents that target estrogen signalling), and biological therapy (e.g. the anti-Her2 monoclonal antibody, trastuzumab).

Endocrine therapy has become the standard of care for ER+ breast cancer, owing to the dependence of these tumours on proliferative signals stemming from ER activity. ER– tumours, in contrast, are intrinsically resistant to endocrine therapy. This became evident in a 1998 overview of clinical trials involving the ER antagonist tamoxifen, in which roughly five years of tamoxifen therapy in ER+ patients was shown to significantly reduce the rate of disease recurrence (from 38.2% to 23.3%) after 10 years of follow-up, whereas ER– patients received virtually no benefit from therapy (67). While tamoxifen remains one of the standard endocrine therapies used today, it carries risks due to its partial agonistic effects in other tissues. In particular, tamoxifen considerably

increases the risk of developing endometrial cancer (68). This has spurred the development of additional agents including the pure estrogen antagonist fulvestrant (ICI 182,780) and a class of drugs known as aromatase inhibitors that prevent the conversion of androgens into estrogen, thereby reducing the concentration of estrogen in post-menopausal women. One such aromatase inhibitor, anastrozole, is possibly superior to tamoxifen (in post-menopausal women) with respect to both efficacy and toxicity profile (69). Although tamoxifen is considered a triumph of targeted cancer therapy, intrinsic or acquired therapeutic resistance remains a significant problem for this modality. Within 15 years following a typical course of tamoxifen therapy, roughly 30% of patients will develop recurrent disease and a quarter will perish (70). The potential causes of endocrine resistance are diverse and will be discussed further in the sections that follow.

Cytotoxic chemotherapies exert their effects by interfering with the process of cell division, typically through one of three broad mechanisms: (a) deprivation of key molecules necessary for DNA replication (e.g. the antimetabolites fluorouracil and methotrexate), (b) direct interaction with DNA and inhibition of DNA replication (e.g. the alkylating agent cyclophosphamide, anthracyclines, and platinum drugs such as cisplatin), and (c) mitotic inhibition (e.g. the microtubule poisons paclitaxel and docetaxel). Because these drugs target all dividing cells, they cause considerable toxicity in any tissues with high levels of cell division, notably the intestines, haematopoietic system, and hair follicles. They are therefore used with caution, their primary contraindications being advanced age, poor baseline health, and low risk of disease recurrence. The most common regimens used today are combinations that include anthracyclines (such as cyclophosphamide, fluorouracil, and either doxorubicin or epirubicin), referred to collectively as anthracycline-based therapies. These improve annual proportional death rates by up to 38%, with the greatest benefit seen in ER– patients (70-73).

Trastuzumab therapy for Her2+ breast cancer is now well established. Trastuzumab is a humanized monoclonal antibody specific to Her2 that functions through inhibition of Her2 signal transduction and by immune sensitization (recognition and destruction of antibody-bound cells by leukocytes). In two major clinical trials, addition of one year of trastuzumab to standard chemotherapy in Her2+ patients led to a relative 48% reduction in recurrence and a 39% mortality reduction compared to chemotherapy alone after four years of follow-up (74).

Due to their lack of specific molecular targets, systemic therapy for triple negative breast cancer (TNBC, meaning clinically negative for ER, PR, and Her2) is restricted to the non-specific cytotoxic chemotherapies described above. TNBC comprises roughly 15% of breast cancers, is

closely associated with the basal-like and claudin-low molecular subtypes, and carries a high risk of recurrence in the first five years following diagnosis (31, 75). Although TNBCs are more sensitive to neoadjuvant chemotherapy (particularly anthracycline-based regimens) than other breast cancers, neoadjuvant-treated TNBCs nevertheless retain a poor prognosis after three to five years (76, 77).

1.3—Estrogen receptor-α (ER) in breast cancer pathogenesis

ER is the currently the most important clinical biomarker for breast cancer and plays key roles during both mammary gland development and tumourigenesis. This section will focus on the biology of ER and its central role in breast cancer endocrine therapy.

1.3.1—ER activity in normal breast development

The importance of ER signalling during mammary gland development has been made clear through the investigation of ER knockout mice. While mammary glands develop normally in $ER^{-/-}$ mice during embryogenesis, no further development occurs during puberty (78, 79). Lack of ER nuclear coregulators results in similar mammary deficiencies. Although estrogen signalling through ER is essential for pubertal mammary outgrowth, it is intriguing that only a minority (up to 30%) of luminal mammary cells actually express ER. It is currently hypothesized that estrogen induces expression of the EGFR ligand amphiregulin in ER+ luminal cells, which then acts in a paracrine manner to stimulate the neighbouring stroma. Both amphiregulin production by mammary epithelial cells and EGFR expression in stromal cells are required for mammary development. Although the actual signal that drives proliferation of luminal mammary cells is unknown, members of the fibroblast growth factor family may fulfill this role (78, 79).

1.3.2—Pathways of ER activation

ER is a steroid receptor member of the nuclear receptor family of transcription factors (80, 81). The functional domain structure of ER is typical of the nuclear receptor family, featuring two central domains involved in DNA binding, dimerization, and nuclear localization; an N-terminal transactivation domain; and a C-terminal ligand binding domain with additional transactivation ability. The classical mechanism of ER activity involves ligand binding, homodimerization, and interaction with specific coactivator or corepressor proteins at conserved DNA sequences known as estrogen response elements (palindromes of two PuGGTCA sites separated by three base pairs). Whether the DNA-bound ER complex triggers transcriptional activation or repression is dependent

on the cell type, the promoter, and the specific ligand. This complexity is evident in the biology underpinning tamoxifen activity—although both tamoxifen and the physiological ligand of ER, 17-β-estradiol, trigger ER dimerization and DNA binding, tamoxifen selectively inhibits transactivation from the C-terminal domain of ER, while the N-terminal domain remains active (81). While this has an attenuating effect on breast tumourigenesis, N-terminal transactivation causes tamoxifen to act as an agonist in endometrial tissue, leading to an increased risk of endometrial cancer (82). Ligand-bound ER can also interact with transcription factors at non-ERE loci. In this situation, ER regulates gene expression not by binding to DNA directly, but by augmenting the activity of the partnering transcription factors, the best characterized of which are AP-1 and SP-1 (83, 84)).

Although ER is primarily localized to cell nuclei, low levels are found in the cytoplasm, where ER can engage in extensive cross-talk with other growth factor pathways. Phosphorylation of ER on Ser^{118} and Ser^{167} by the Ras-ERK (extracellular regulated kinase) and PI3K (phosphatidylinositol-3-kinase)-Akt pathways, respectively, can result in ligand-independent activation of ER (85, 86). Conversely, ligand-bound ER can participate in protein complexes that promote activation of Akt and ERK signalling. Estrogen stimulation of MCF7 breast carcinoma cells results in rapid and transient activation of the mitogen-activated protein kinase (MAPK) cascade that is dependent on ligand-bound ER (87). This appears to occur through an estrogen-dependent interaction of ER with the growth factor receptor adaptor protein Shc that triggers downstream MAPK signalling (88). Along with phosphorylation, ligand-bound ER can also be methylated by the arginine methyltransferase PRMT1. This triggers the formation of a complex incorporating ER, PI3K, Src, and focal adhesion kinase (FAK) that ultimately causes activation of Akt (89). Others have also noted a functional interaction between ER and PI3K (90). Yet another complex involves the ER coactivator PELP1. When localized to the cytoplasm, PELP1 can act as a scaffold to promote ER interaction with Src, leading to MAPK activation (91). Finally, ligand-bound ER can promote the activation of growth factor receptors such as EGFR, Her2, and insulin-like growth factor-1 receptor (IGF-1R (92-94)). The various mechanisms of ER functionality are summarized in Figure 2.

ER activation in breast tumour cells ultimately causes complex changes in gene expression that primarily mediate increased proliferation, but can also influence cell migration and survival. ER-regulated genes fall into several functional categories (95) including cell cycle machinery (e.g. cyclin D1 (induced)), apoptosis regulation (e.g. survivin (induced) and caspase-9 (suppressed)), growth factor ligands (e.g. amphiregulin (induced)), signal transduction proteins (e.g. JAK1 (induced)

and Her2 (suppressed)), and transcription factors (e.g. c-fos, c-myc, and c-myb (induced)). Given the intersection of ER with diverse pathways that are vital to tumourigenesis, it is unsurprising that endocrine therapies have become highly successful clinical strategies. Less clear is why the expression of ER and ER-regulated genes correlates with favorable pathological features and good prognosis (15, 31). This apparent paradox (i.e., that ER promotes tumourigenesis and is an excellent therapeutic target, yet is also a good prognostic marker) is not currently understood.

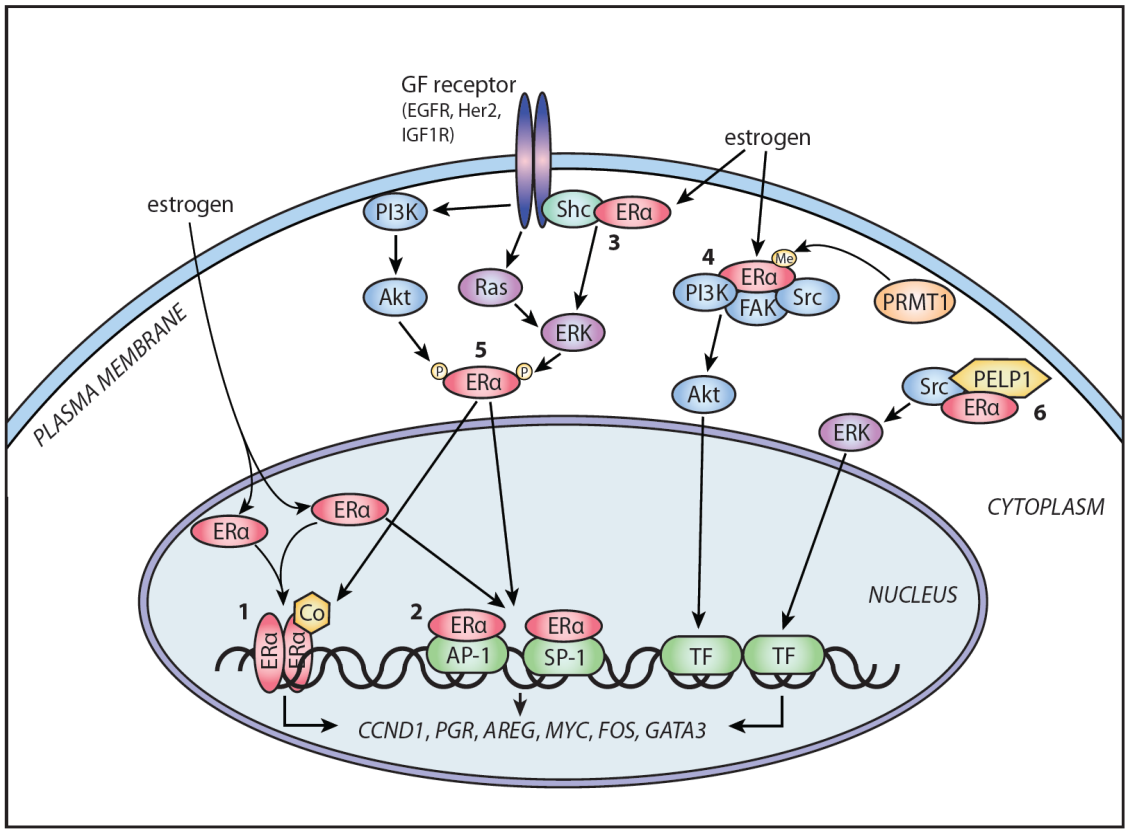

Figure 2. **Mechanisms of estrogen receptor-α (ER) signalling**. ER regulates gene expression and signal transduction via multiple mechanisms, identified in this figure by the numbers 1 to 6. (**1**) Interaction of ER with agonists such as 17-β-estradiol causes homodimerization and DNA binding at specific DNA sequences known as estrogen response elements (EREs). The resulting stimulation or suppression of target gene transcription is determined in part by the cofactors (Co) that interact with DNA-bound ER. (**2**) Alternatively, ligand-bound ER can influence gene expression through modulation of other transcription factors such as AP-1 and SP-1. (**3**) In the cytoplasm, ligand-bound ER can interact with Shc to promote ERK activation, as well as promote Akt activity (**4**) through participation in a complex involving PI3K, Src, and FAK that is formed following methylation of ligand-bound ER by PRMT1. (**5**) In the absence of ligand, ER can be phosphorylated and activated by ERK and/or Akt as a consequence of growth factor receptor signalling. (**6**) In a ligand-independent fashion, cytoplasmic ER can form a complex involving its cofactor PELP1 and Src to promote ERK signalling.

1.3.3—ER suppression and resistance to endocrine therapy

As we have described above, approximately one third of breast cancers are clinically ER– at diagnosis and will not receive benefit from endocrine therapy. The lack of ER is referred to as an intrinsic resistance mechanism. Acquired resistance, by contrast, refers to the recurrence of tumours that were originally responsive to endocrine therapy. While most of these recurrent lesions maintain ER expression, roughly 20% lose ER over time and exhibit estrogen-independent growth (96). In general, investigations of endocrine resistance have focused on mechanisms that involve maintenance of ER expression. Many such mechanisms are proposed, most of which are based on changes of expression and/or function of other proteins involved in ER activation and signalling (96). These include overexpression of ER transcriptional coactivators, overexpression of alternative transcription factors with which ER interacts (e.g. AP-1), overexpression or hyperactivation of growth factor receptors such as Her2, and changes in the local microenvironment that influence growth factor signalling. The impact of Her2 expression on tamoxifen resistance was made clear using a mouse xenograft model of Her2-overexpressing MCF7 cells (94). Like wild type MCF7s, these cells require ER activity for *in vivo* proliferation. However, while tamoxifen inhibits tumour growth in wild type cells, it acts as a potent agonist in those engineered to express Her2. This is because tamoxifen-bound ER is capable of activating Her2/EGFR signalling. ER is in turn activated by Her2/EGFR via phosphorylation, establishing cross-talk that potently drives tumourigenesis in response to tamoxifen. Based on this premise, therapies involving combinations of endocrine disruptors and EGFR family inhibitors such as gefitinib are currently being tested in the clinic.

While much effort has been made to understand the rewiring of ER signalling that takes place during acquisition of endocrine resistance, relatively little has been devoted to the issue of ER loss with progression. We are similarly poor at explaining why 30% of breast tumours are ER– at clinical presentation. As discussed in Section 1.2.2, one hypothesis is that ER– tumours arise from an ER– precursor cell and, similarly, that ER+ tumours arise from ER+ precursors. This seems unlikely, however, given that less than a third of luminal epithelial cells in the normal breast are ER+ at any one time and, since these cells are almost never dividing (97), would not be expected to accumulate the substantial mutations necessary to form a tumour. In addition, roughly 5% of ER+ *in situ* carcinomas have associated invasive components that are ER– (97). Perhaps most importantly, considerable phenotypic heterogeneity exists within individual tumours. Allred *et al* (98) clearly demonstrated that many *in situ* ductal carcinomas are composed of regions with differing

histological grade and biomarker status (including ER). Thus, although a tumour's cell of origin may have influence on whether it expresses ER, it is likely that other variables are also important.

Several mechanisms of ER regulation in breast cancer have been reported, with varying degrees of supporting clinical evidence. Ligand-binding induces rapid proteolysis of ER (99), as well as transient suppression of *ESR1* mRNA levels (100). However, despite this transient downregulation, ER remains readily detectable following estrogen stimulation of breast tumour cells. A key transcription factor that appears to be required for ER expression is GATA3. In ER+ breast tumour cells, GATA3 and ER maintain each other's expression in a positive regulatory loop (101). Furthermore, GATA3 is positively associated with ER in breast cancer and is a defining feature of luminal-subtype breast tumours (102). In contrast, c-Jun overexpression and increased AP-1 activity can lead to ER suppression and estrogen non-responsiveness (103). Changes in either GATA3 or AP-1 function could thus impinge on ER expression. Overexpression of the EMT transcription factor snail has also been shown to reduce ER expression (104). Somewhat surprisingly, no conclusive data suggests that ER expression is lost due to mutation or genomic aberrations (97).

Several studies have suggested that ER-negativity may result from suppressive epigenetic modifications of the *ESR1* locus. Abnormally high methylation of the *ESR1* promoter has been observed during breast cancer progression and is associated with ER– tumours (105, 106). Furthermore, it is possible to restore ER expression in ER– MDA-MB-231 breast cancer cells by treating them with inhibitors of histone deacetylases and/or DNA methyltransferases (107, 108). While the mechanisms underlying these observations are unclear, a recent study indicated that IL-6 treatment of ER+ MCF7 cells could induce *ESR1* promoter methylation and reduction of ER expression. Conversely, abrogation of autocrine IL-6 signalling in MDA-MB-231 cells resulted in reduced *ESR1* methylation and increased ER expression (109). Thus, microenvironment factors such as cytokines may mediate reversible ER suppression via epigenetic regulation of *ESR1*.

Current evidence appears to support a key role for the tumour microenvironment in regulating ER. Hypoxia, a common and dynamic stress factor within the disorganized tissue architecture of tumours, has been shown by several groups to suppress ER expression at both the protein and mRNA level (110-113). In clinically ER+ breast cancer tissues, ER expression is often reduced or absent in hypoxic areas (110). Hyperactive growth factor signalling is perhaps the best characterized mechanism of ER suppression. MCF7 breast carcinoma cells engineered to express constitutively active Raf kinase or high levels of EGFR or Her2 lose ER expression and estrogen sensitivity in an MAPK-dependent manner (114). Gene expression signatures derived from these

cells demonstrate that many ER– tumours exhibit MAPK-driven expression patterns (115). Similarly, protein and mRNA expression patterns indicative of PI3K signalling were found to be elevated in human ER+ breast tumours with reduced ER expression (116). Finally, MAPK inhibitors can restore ER expression in some ER– cell lines and primary tumour explants (117). Clinically, increased EGFR/Her2 expression is often seen in breast tumours with acquired tamoxifen resistance, and some early-phase clinical trials have demonstrated that blockade of EGFR/Her2 in combination with endocrine therapy may be beneficial (96, 118). As already alluded to, cytokines (primarily IL-6, IL-1, and TNF) can also negatively regulate ER (109, 119, 120). However, other studies have paradoxically reported the ability of each of these cytokines to promote ER-dependent transcriptional activity (121-123). Nevertheless, the high levels within ER– tumours of cytokines and infiltrating leukocytes (from which many cytokines are likely derived (124, 125)) supports a potential role for cytokine signalling as a regulator of ER expression in breast cancer. Continued investigation of the breast cancer microenvironment may yield crucial insights into the etiology and regulation of ER expression and the clinical and biological disease subtypes with which it associates.

1.4—The Janus faces of tumour immunology

A key component of the tumour microenvironment is the highly variable repertoire of leukocytes that initially infiltrate lesions in response to perturbations in tissue homeostasis. The association between tumours and hematopoietic cells was recognized as early as 1863, when Virchow postulated that cancer arises at sites of chronic inflammation (126). Although the field of tumour immunology remained obscure in the century following Virchow's observations, it now enjoys widespread recognition as an integral aspect of cancer biology. Tumour immunity is frequently described as a 'double-edged sword' due to the paradoxical ability of leukocytes to both eradicate and provide succor to tumours. The following sections will briefly explore these contrasting themes.

1.4.1—The good: host-protective effects of anti-tumour immune responses

Tumour-infiltrating leukocytes (TIL) include cells of both the myeloid and lymphoid lineages. In general, macrophages are the major intratumoural representatives of the myeloid lineage, followed by granulocytes and, rather more sporadically, dendritic cells. Tumour-infiltrating lymphocytes include T cells, B cells, and natural killer (NK) cells. The presence of leukocytes in tumours is thought to reflect an attempt by the immune system to eradicate the neoplasm, and certain subsets

of leukocytes, particularly cytotoxic T cells and NK cells, clearly have the ability to do so (127). As such, the theory of immune surveillance postulates that the vast majority of malignant cells are eradicated by the immune system before they can manifest clinically detectable lesions (128). Indeed, in mice with occult carcinogen-induced sarcomas, ablation of adaptive immune components such as T cells dramatically increases the outgrowth of overt tumours (129). Furthermore, the presence of tumour-infiltrating leukocytes in multiple human tumour types is reproducibly associated with good prognosis, suggesting that leukocytes can continue to exert anti-tumour effects even in the context of full-blown disease (130-137).

Mobilization of an organized anti-tumour immune response requires recognition of tumour antigens by lymphocytes. Because lymphocytes are selected during development to be non-responsive (tolerant) toward normal host molecules, tumour antigens must be different in such a way that they can be specifically recognized as foreign. Often this occurs through mutation of a protein or, alternatively, overexpression of a non-mutated tumour antigen (e.g. Her2 in breast cancer) at levels high enough to overcome normal tolerance mechanisms (127). While tumour-reactive B cells can hobble tumours with antigen-specific antibodies, antigen-specific cytotoxic T cells (widely considered to be the primary agents of anti-tumour immunity) can directly interact with antigen-bearing tumour cells and induce their apoptotic death, usually through mechanisms including perforin/granzyme secretion and ligation of the death receptor Fas (138).

Exploitation of anti-tumour immunity for therapeutic purposes is becoming rapidly recognized as a promising area of discovery. The first therapeutic cancer vaccine (139), sipuleucel-T, was approved in 2010 for use against metastatic prostate cancer and has been hailed as a landmark for the field of cancer immunotherapy. Another strategy is known as adoptive T cell therapy (140). This involves the resection of a patient's tumour, *ex vivo* growth and propagation of the tumour-reactive T cells found within, and re-infusion of the expanded T cell population (generally 10 to 100 billion cells) intravenously into the patient. The re-infused cells traffic naturally to remaining tumour beds to exert cytotoxic effects on target cells. This is currently the most effective therapeutic option for eligible metastatic melanoma patients. In addition, genetic programming of T cells to recognize specific tumour antigens is under investigation in several laboratories worldwide, raising the possibility of cancer treatment via rationally designed and individualized cellular therapies.

The immune system may also play a vital role in conventional cancer therapy. It has been known since the 1950s that irradiation of a single tumour can inhibit the growth of secondary tumours that reside beyond the radiation field, a phenomenon known as the abscopal effect (141).

We now know that this is at least partially immune-mediated, in that cancer cells killed by radiation appear to elicit immune responses that can suppress tumour progression elsewhere in the host (142, 143). Certain chemotherapies may work in a similar fashion. Drugs in the anthracycline family can induce a form of apoptosis that triggers immune recognition of dying tumour cells (144). Emerging data from studies of human cohorts support this premise (145). For example, we have recently shown that infiltration of ER– breast tumours by cytotoxic T cells prior to treatment is strongly associated with favourable responses to anthracycline-based chemotherapy (146).

1.4.2—The bad: tumour immune escape and exploitation

Cancer exists despite immune surveillance, demonstrating the ability of tumours to evade destructive immune responses. Immune escape can occur through many possible mechanisms, including downregulation of antigen presentation or outgrowth of antigen loss variants, functional exhaustion of tumour-reactive T cells, induction of immune tolerance mechanisms, or direct tumour-mediated suppression of immune activity (for example, production of immunosuppressive cytokines such as TGFβ). Our increasing awareness of these processes has led to the addition of tumour immune escape to the list of cancer hallmarks proposed by Hanahan and Weinberg, published originally in 2000 and updated in 2011 (147, 148).

More insidious than immune escape is the process by which leukocytes become active participants in tumorigenesis (the dark edge of the proverbial sword). Rather than simply evolving the means to develop in the face of immune pressure, many tumours manifest additional mechanisms that allow them to exploit immune responses (149, 150). Epidemiological evidence to support this premise includes the association of certain tumour types with chronic inflammation (such as the predisposition of patients with inflammatory bowel disease and prostatitis to develop colorectal and prostate cancer, respectively (151)) and the reduced risk of cancer in individuals who regularly use non-steroidal anti-inflammatory drugs (e.g. aspirin) or selective inhibitors of cyclooxygenase-2, a key enzyme of the inflammatory cascade (152).

Immune responses to conventional infectious targets are typically acute, in that leukocytes arrive at the site of infection, eradicate the offending target, repair damaged tissue, and ultimately vacate. In contrast, tumours often appear to harbour chronic or 'smoldering' inflammation, in which leukocytes infiltrate the tumour and exist in varying states of activation that fail to resolve (151). Tumour-associated macrophages (TAMs) are particularly notorious in this context (153-155). Macrophages display a great deal of phenotypic plasticity and have the capacity to promote

destructive immune responses during acute stages of inflammation (known as M1 polarized macrophages), as well as dampen inflammation and promote wound healing during resolution of immune responses (M2 macrophages (156)). Functionally speaking, TAMs generally exhibit an M2-like phenotype: they promote extracellular matrix remodeling through expression of various proteases, produce growth factors and cytokines that support cell survival and proliferation, and encourage angiogenesis through production of vascular endothelial growth factor (VEGF). In addition, TAMs can impair adaptive anti-tumour immunity through secretion of immunosuppressive cytokines, recruitment of regulatory T cells (Tregs), and production of the enzymes arginase and indoleamine 2,3-dioxygenase (IDO) which respectively deplete arginine and tryptophan, two essential amino acids for lymphocyte activation. These activities translate into a consistent clinical association between high TAM levels and poor patient prognosis in a variety of human tumour types, including breast cancer (154, 157).

Unlike TAMs, tumour-infiltrating lymphocytes ((TIL) such as T and B cells) are viewed more favourably as deterrents of cancer progression. Nevertheless, various lymphocyte subsets have been associated with poor clinical features in multiple tumour types and have been directly demonstrated to support tumourigenesis in experimental models (150, 158, 159). Tregs provide the most obvious example: these cells are vital to the maintenance of self-tolerance in normal tissue settings and can be recruited by tumours to suppress potentially destructive anti-tumour immune responses (160-162). As such, although a lack of Tregs in mice and humans leads to lethal autoimmunity, their presence in tumours is associated with poor prognosis in diseases such as breast and ovarian cancer (161, 163-165). In murine breast cancer models, $CD4^+$ (helper) T cells have been shown to promote metastasis indirectly through induction of EGF production by TAMs (166). Similarly, $CD8^+$ (cytotoxic) T cells can induce breast cancer cells to undergo EMT and acquire stem cell-like features, possibly through production of TNF (57, 60). Breast cancer cells with a $CD44^{high}CD24^{-/low}$ phenotype (putative cancer stem cells) were recently shown to express elevated levels of the chemokine CCL5, a potent chemoattractant for T cells, suggesting that breast cancer stem cells may actively recruit lymphocytes to the tumour bed (167).

Clinically, intratumoural lymphocytes in human breast cancer are associated with aggressive pathological features such as high tumour grade and ER– status (125, 157, 164, 168-170), and are a notable feature of basal-like (and/or claudin-low) breast cancers, which have high rates of metastasis and are thought to contain relatively high levels of putative cancer stem cells (23, 54, 171, 172). Limited evidence suggests that TIL may be a negative prognostic marker for ER+ breast cancer

(164, 170). Given these observations, it is curious that multiple studies have shown a strong relationship between TIL and favorable prognosis in high-risk breast cancers (e.g. ER–, Her2+, or highly proliferative lesions (130-132, 170, 173, 174)). Though largely speculative, it is possible that these data can be reconciled by envisioning a breast cancer risk continuum based on tumour inflammation. In this model, early stage breast cancers that avoid immune destruction may contain TIL that are either functionally perturbed or at levels too low to curtail tumour progression—in this setting, sub-optimal TIL activity could be exploited by the tumour, causing it to progress to a more aggressive phenotype (explaining the association between TIL and aggressive tumour subsets). However, some of these tumours may eventually lose control of anti-tumour immunity, leading to heightened TIL levels and functionality which (with the help of therapeutic intervention) could increase the likelihood of tumour regression and cure (thus explaining the link between very high TIL levels and good outcome in what should otherwise be poor-prognosis breast tumours).

Given that immune exploitation by tumours appears to occur frequently and may contribute significantly to the generation of dangerous tumour phenotypes, it is increasingly believed that intervention in this process could be therapeutically profitable. Less frequently acknowledged is the risk that immune exploitation could hamper efforts to therapeutically enhance anti-tumour immunity. A broad understanding of the mechanisms by which tumours profit from immunity could thus provide not only novel therapeutic targets, but also a roadmap of the potential pitfalls that should be avoided (or at least accounted for) when designing and implementing cancer immunotherapies.

1.5—Oncostatin-M and related cytokines

Cytokines are crucial to the orchestration of immune responses. As such, the cytokine milieu is a highly variable component of the tumour microenvironment that depends on the type, quantity, and functional status of infiltrating leukocytes. Additional sources of cytokines in breast tumours include fibroblasts, adipocytes, and the malignant cells themselves (124, 158, 175). Three prominent cytokines that are produced during early stages of inflammation are IL-1, IL-6, and TNF. These are typically produced following the activation of tissue-resident leukocytes by molecules derived from pathogens or damaged host tissue, and are essential for establishing local inflammation through induction of leukocyte chemoattractants, activation of endothelial (blood vessel) permeability, and induction of matrix remodeling enzymes that facilitate leukocyte invasion. In tumours, unfortunately, these processes can aid the dissemination of neoplastic cells. Furthermore, these

cytokines can stimulate malignant cells directly, triggering survival and invasion mechanisms via signal transduction through the STAT3 (signal transducer and activator of transcription 3), NF-κB (nuclear factor kappa-B), and MAPK pathways (176). Relative to normal breast tissue (124), many other cytokines are prevalent in tumours, including the chemokines IL-8, CCL2, and CCL4, which collectively attract both lymphocyte and myeloid cell types; IL-2 and interferon-γ (IFNγ), key agents of T cell activation and cell-mediated immunity; and IL-4 and IL-13, which promote B cell-mediated immunity. The following sections are devoted to discussion of the IL-6 family, with particular emphasis on OSM.

1.5.1—Overview of the IL-6 cytokine family

IL-6 is the prototypic member of a pleiotropic cytokine family that includes OSM, leukemia inhibitory factor (LIF), IL-11, ciliary neurotrophic factor (CNTF), cardiotrophin-1, cardiotrophin-like cytokine, and neuropoietin (177). These cytokines are united by the shared requirement for gp130 in their receptor complexes and, as such, have considerably overlapping functions. Expression of gp130 occurs ubiquitously in tissues and is a vital developmental factor: $gp130^{-/-}$ mice begin to die 12.5 days post-coitum with a wide range of defects, including impaired heart development and hematopoiesis (178). Tissue specificity for recognition of distinct cytokines is based on interaction of gp130 with specific secondary receptor chains including IL6R, LIFR, and OSMR. IL-6 binds to a complex containing two gp130 chains and one IL6R chain (which provides specificity for IL-6 but does not contribute to signal transduction). Signalling from gp130 occurs as follows: ligand binding triggers activation of gp130-associated janus kinases (JAKs) that phosphorylate tyrosine residues on gp130. This leads to recruitment of STAT proteins (particularly STAT3) to gp130 via their SH2 domains. STATs are phosphorylated in turn by JAKs, leading to STAT homodimer formation, nuclear translocation, and regulation of gene expression. In addition to STATs, ligand binding triggers recruitment of the adaptor protein SHP2 to gp130, which leads to activation of the Ras-Raf-MAPK pathway. Finally, through a poorly understood mechanism, IL-6 family members can also trigger activation of the PI3K-Akt pathway (179). Mice deficient in IL-6 show defects in hematopoiesis and immune responses including antibody production and recruitment of leukocytes to inflamed areas. $LIFR^{-/-}$ mice display embryonic lethality due to placental defects and loss of motor neurons (177). OSMR, in comparison, appears less vital; $OSMR^{-/-}$ mice are viable and fertile, but display mild deficiencies in production of red blood cells and platelets (180) and are less capable of healing tissue damage in the heart and liver (181, 182).

1.5.2—Roles of IL-6 in breast cancer

IL-6 is one of the best studied cytokines with respect to its roles in breast cancer. Although data concerning the actual levels of IL-6 in breast tumours are surprisingly scarce, Chavey *et al* (124) convincingly demonstrated that breast tumours frequently contain high concentrations of IL-6 protein relative to normal breast tissue. Furthermore, they show that its expression is particularly high in tumours with unfavourable clinical features such as ER-negativity and high grade. Several groups have measured IL-6 in human serum and observed high levels in the blood of breast cancer patients versus healthy controls. Serum IL-6 also appears to be positively correlated with advanced disease stages and poor prognosis (183). Although intratumoural leukocytes are an important source of IL-6 in breast cancer, IL-6 can also be produced by malignant breast cells. This is exemplified by the MDA-MB-231 breast carcinoma cell line, which maintains ER suppression via autocrine IL-6 production (109).

IL-6 appears to have several effects on breast cancer cells that collectively implicate it as a promoter of disease progression. For example, it has been shown to induce cell migration (184), resistance to chemotherapies (185), and EMT (186). IL-6 can also promote invasiveness and mammosphere formation in MCF7 cells, and is expressed at high levels in mammospheres derived from primary human breast tumours (61). Consistent with this, IL-6 promotes the generation of a $CD44^{high}CD24^{low/-}$ breast cancer cell population (putative cancer stem cells) with enhanced tumourigenicity in mice (187). Various *in vivo* models support these data—inhibition of gp130 signalling in MDA-MB-231 cells strongly impairs their ability to form tumours in athymic nude mice (188), and IL-6 appears to be required for efficient tumourigenesis in both Her2- and Ras-driven breast tumour models (189, 190). Despite these data, clinical trials of IL-6-targeted breast cancer therapies have yet to occur.

1.5.3—Normal and pathological roles of OSM

OSM was originally identified in 1986 as a factor produced by histiocytic lymphoma cells that exerted potent cytostatic effects on A375 melanoma cells and other cancer cell lines (hence the name oncostatin-M (191)). Although it has not enjoyed the popularity of cytokines such as IL-6 and TNF, OSM is nevertheless recognized for its diverse array of biological roles in both normal and pathological processes (192). Unlike cytokines such as IL-6, cellular sources of OSM appear to be exclusively hematopoietic (with the possible exception of osteoblasts); specific examples include T cells (193), dendritic cells (194), macrophages (195), neutrophils (196), and mast cells (197). OSM is

unique among IL-6 family members in that it can functionally interact with two distinct receptor complexes: heterodimers of either gp130-LIFR or gp130-OSMR. Although this has not been formally demonstrated, OSMR appears to have either a wider tissue distribution or is expressed at higher levels in many tissues relative to LIFR, since several studies have described various OSM functions as being mediated primarily through gp130-OSMR complexes. Unlike IL6R, OSMR can transduce signals along with gp130. Interestingly, rather than using SHP2 to trigger MAPK signalling, OSMR employs the adaptor protein Shc (179).

The role of OSM during inflammatory responses is somewhat controversial, as it shows both pro- and anti-inflammatory effects in a context-dependent manner. Exposure of endothelial cells to OSM results in potent induction of P- and/or E-selectin expression that triggers neutrophil adhesion, rolling, and transendothelial migration (198, 199). Interestingly, OSM may be more effective in this role than its family members IL-6, IL-11, and LIF. Stimulation of human monocyte-derived dendritic cells with bacterial products causes production of high levels of OSM (194) and OSM in turn may cause enhanced homing of dendritic cells to regional lymph nodes (200). Studies of skin inflammation have shown that OSM production by T cells in psoriasis and dermatitis lesions contributes to keratinocyte hyperplasia and expression of additional pro-inflammatory and defensive factors (201-203). Similarly, OSM is highly expressed by infiltrating neutrophils in response to skin wounding (204) and has also been shown to promote inflammation and fibrosis of pulmonary tissue (205, 206). In contrast, OSM production by neutrophils during early stages of peritonitis may dampen inflammation by inhibiting further recruitment of innate leukocytes (196, 207).

Arthritis is the primary inflammatory disease in which OSM is implicated. Under normal conditions, OSM appears to regulate bone turnover in mice, in part by inducing RANKL (receptor activator of nuclear factor kappa-B ligand)-mediated osteoclast differentiation (208). In models of inflammatory arthritis, overproduction of OSM elicits RANKL production and pathological bone destruction, particularly in the presence of TNF or IL-1 (209). OSM appears to have similar destructive effects on cartilage (210). When compared with IL-6, OSM induced markedly higher levels of joint inflammation and tissue damage (211). Finally, administration of OSM-neutralizing antibodies to mice with collagen-induced arthritis was shown to strongly inhibit the progression of disease (212). OSM has also been implicated in the pathogenesis of multiple sclerosis (MS), having been identified as a factor produced spontaneously at high levels by peripheral blood leukocytes from MS patients (213). This is supported by the observations that prostaglandin-E2 causes OSM

production in microglia (nervous system macrophages (195)) and that OSM induces production of TNF by microglia that can mediate neuronal cell death (214).

Beyond inflammatory processes, OSM can support tissue integrity in multiple settings. In a rat model of retinal degeneration, OSM treatment protected mice from loss of rod cells (215). OSM also appears to be necessary for the development of nociceptive neurons, in that $OSM^{-/-}$ mice are much less sensitive to acute pain (216). OSM can be used *in vitro* to induce maturation of primitive hepatocytes (217), and OSM produced from Kupffer cells (liver macrophages) was necessary for efficient liver recovery after physical or chemical-induced injury in mice (181). Finally, OSM was recently shown to support heart recovery after myocardial infarction, apparently by inducing dedifferentiation of cardiomyocytes (182).

Despite its initial discovery as a cytostatic factor for melanoma cells, the roles of OSM in cancer are not well defined. Nevertheless, the available literature suggests that OSM may be involved in the pathogenesis of diverse tumour types. A single report on lung cancer cells described the ability of OSM to induce CD44 expression (218), which is intriguing in light of the well-documented link between CD44 expression and heightened malignancy (219). OSM treatment of $OSMR^{+}$ cervical squamous carcinoma cell lines was shown to induce migration, invasiveness, and a pro-angiogenic phenotype (220). Similar results were obtained with osteosarcoma cell lines (221). However, OSM has also been shown to sensitize osteosarcoma cells to apoptosis (222). Importantly, the link between OSM and cervical cancer was supported clinically in a study that identified gene copy number gain of the *OSMR* locus in over 60% of human cervical cancers, a feature that was significantly associated with poor prognosis (223). OSM may also be involved in the pathogenesis of hematopoietic malignancies: an activating mutation in JAK2 (V617F) that occurs frequently in myeloproliferative neoplasms (MPN) was found to drive substantial OSM expression in MPN cells, which could then exert various tissue remodeling effects on cells of the bone marrow (224). Prostate cancer may also be exacerbated by OSM signalling, as OSM has been shown to induce ligand-independent activation of the androgen receptor in prostate carcinoma cells (a similar phenomenon to growth factor-induced ER activation in breast cancer (225)). In contrast to these findings, studies of colon and liver cancer suggest that OSM may have protective effects in these settings. Consistent with the differentiating effect of OSM on normal hepatic progenitor cells, Yamashita *et al* (226) recently reported that OSM can promote differentiation of putative cancer stem cells in hepatocellular carcinomas, leading to enhanced tumour susceptibility to treatment with the chemotherapy drug 5-fluorouracil. In the intestinal setting, two studies have demonstrated that

methylation and silencing of the *OSMR* gene may occur in up to 80–90% of primary colon tumours (227, 228). Restoration of OSMR expression in colon cancer cells using DNA methyltransferase inhibitors rendered them sensitive to OSM-mediated growth suppression (228). Notably, these authors also observed *OSMR* methylation in gastric and pancreatic cancers, but not cancers of non-digestive organs, suggesting that OSMR suppression may be particularly important for digestive system malignancies (227).

Several studies have addressed the effects of OSM on breast cancer cells. Early studies that took place in the 1990's and early 2000's focused largely on the cytostatic impact of OSM which, depending on dose, treatment duration, and the cell line used, could reduce cell proliferation by up to 90% (229-232). This was partly attributed to OSM's ability to suppress expression of the proto-oncogene c-myc (230, 231). OSM was also shown to inhibit expression of the p53 tumour suppressor, but the functional consequences of this were not explored (233). Grant *et al* (234) made an additional intriguing observation in 2002: despite the well known mitogenic effects of EGF on breast tumour cells, EGF treatment enhanced the cytostatic effect of OSM. They went on to reveal that both gp130 and OSMR physically associate with EGF family receptors such as Her2, and that this interaction mediates signal transduction cross-talk between the two pathways. Because of the consistent observation that OSM suppressed breast cancer cell proliferation, it was argued that OSM could be exploited as a novel biological therapy for breast cancer.

Starting in 2003, the concept that OSM could be used therapeutically began looking less attractive. Zhang *et al* reported that while the strong STAT3 activation elicited by OSM was responsible for its growth-suppressive properties, STAT3 signalling was also a major driver of cell migration (235), consistent with prior (but generally overlooked) observations that OSM caused loosening of cell-cell junctions and loss of normal epithelial morphology. As an intriguing aside, Kaposi sarcoma is a rare example of a tumour that increases proliferation in response to OSM. This appears to be the result of STAT3 suppression by a modified cyclin protein encoded in the genome of the Kaposi sarcoma-associated herpesvirus (236). Holzer *et al* expanded the study of OSM-induced migration using murine mammary carcinoma cells—as with human cells, OSM suppressed proliferation, but also enhanced metastatic properties including detachment from the substrate (with maintenance of viability) and invasiveness through matrigel (237). Jorcyk *et al* observed the same pro-invasive effect of OSM using human T47D breast carcinoma cells (238). Finally, Queen *et al* (239) demonstrated that neutrophils can release OSM upon contact with human MDA-MB-231 or T47D breast cancer cells. Intriguingly, this was dependent on direct contact between neutrophils and

tumour cells, as well as tumour cell secretion of GM-CSF (granulocyte/macrophage colony stimulating factor). Functional consequences of this interaction included increased tumour cell detachment, invasiveness, and expression of VEGF, all of which could be inhibited by neutralizing antibodies to OSM or blocking antibodies to OSMR. Collectively, the studies of the past decade suggest that while OSM may inhibit cell proliferation, this is countered by the promotion of metastatic properties. Given that metastasis is by far the primary cause of breast cancer mortality, these data imply that OSM may have deleterious effects as a therapeutic agent, and could instead be considered a plausible therapeutic target.

It should be noted that OSM is known to have a key role in the normal mammary gland. Lactation in both mice and humans is associated with pronounced outgrowth of mammary parenchyma to support the feeding of offspring. Following weaning, this process is reversed through apoptosis of mammary epithelial cells and tissue remodeling (known as involution). OSM has been shown to be a key mediator of post-lactational involution in mice, with direct pro-apoptotic effects on mammary epithelial cells (240). A recent study has shown that in normal human mammary epithelial cells (HMECs), OSM delivers a potent cytostatic effect that is dependent on STAT3-mediated suppression of c-myc (241). Constitutive expression of c-myc in these cells largely prevented OSM-mediated growth suppression, and rendered OSM a potent stimulant of anchorage-independent growth. These data suggest that OSM normally has a restraining effect on mammary epithelial cells, but under conditions of deregulated growth factor pathways (as occurs in tumours), this effect is overridden, turning OSM into a potentially oncogenic stimulus. Despite the potential role of OSM in breast cancer progression, the clinical relationship between OSM signalling and breast cancer outcome has not been explored. Notably, loss of SOCS3 (suppressor of cytokine signalling-3), a key negative regulator of IL-6 family receptors, was recently shown to be a strong predictor of poor prognosis in human breast cancer (242). However, since the effects of SOCS3 are not restricted to OSM signalling, the relationship between OSM and breast cancer prognosis remains an open question.

1.6—S100A7 (psoriasin) in cancer and inflammatory disease

S100A7 is one of over 20 members of the vertebrate-specific S100 family. S100 proteins are 10–12 kDa in size and possess two calcium-binding EF-hand motifs. S100 proteins typically exist as non-covalent anti-parallel homo- or heterodimers that undergo conformational changes in response to calcium binding, causing exposure of interaction sites that permit binding and modulation of target

proteins (243, 244). In this manner they are thought to act as molecular switches that regulate cellular behaviour in response to local calcium levels. Like cytokines, S100 proteins exert numerous functions in different tissues, including regulation of cell growth, motility, and survival.

1.6.1—Expression and function of S100A7 during inflammation

S100A7 is expressed normally during differentiation in epidermal keratinocytes (245), where it acts as an innate defensive protein against bacteria (particularly *Escherichia coli* (246, 247)). S100A7 was initially discovered as a highly expressed gene in psoriatic skin (hence the alternative name 'psoriasin' (248)), and has since been observed as an upregulated factor in several inflammatory skin disorders (244). Various cytokines are known to induce S100A7 expression in skin, including OSM (201-203), IL-6 (201), IL-1 (201, 249, 250), TNF (201, 203), IL-22 (201, 203, 251-254), and IL-17 (201, 203, 252). In conjunction with IL-1, ligands of EGFR may also induce S100A7 (250).

The specific effects of S100A7 during inflammation remain poorly characterized. S100A7 is known to be secreted (255), raising the possibility that it could exert extracellular effects in the inflammatory environment. Indeed, two studies have shown S100A7 to be a leukocyte chemoattractant (256, 257), potentially by interacting with the pattern recognition receptor RAGE (receptor for advanced glycation end products). Recombinant S100A7 has also been shown to induce expression of the chemokines IL-8, CXCL10, and CCL20 in human keratinocytes (258). Similarly, S100A7 stimulation of neutrophils was shown to trigger secretion of IL-6, TNF, IL-8, CCL3, CCL4, and CCL20 (259). Thus, in addition to exerting microbicidal functions, S100A7 may directly regulate immune responses through its effects on epidermal cells and leukocytes.

1.6.2—S100A7 biology in breast cancer

S100A7 was identified as a highly expresssed factor in breast cancer in the mid-1990s (260, 261). This was subsequently confirmed by a proteomics screen that identified S100A7 as one of the most abundant proteins in DCIS lesions relative to normal breast tissue (262). Importantly, no studies have demonstrated significant S100A7 expression in normal tissue. Attempts to determine the origins of its expression in cancer revealed that BRCA1 and c-myc can cooperatively repress S100A7 (263), while estrogen stimulation may induce S100A7 via estrogen receptor-β ((ERβ) (264)). The only other study to address this question revealed that general stresses such as serum deprivation could also increase S100A7 in cultured mammary cells (265). As such, there is considerable scope to explore the mechanisms of S100A7 regulation in breast cancer.

Multiple studies implicate S100A7 as a driver of breast cancer progression. Animal models have shown that overexpression of S100A7 in MDA-MB-231 xenografts promotes tumour incidence and growth rate (266). Similarly, shRNA-mediated knockdown in the endogenously S100A7+ MDA-MB-468 cell line reduces *in vivo* tumourigenesis (267, 268). A key mechanism by which S100A7 exerts oncogenic effects appears to be the intracellular modulation of Jab1 (CSN5). Jab1 is an important catalytic subunit of a larger protein complex called the COP9 signalosome that regulates the activity of cullin-based E3 ubiquitin ligases (collectively, these control the stability of a large number of proteins with known roles in cancer (269)). Intriguingly, Jab1 is also a key mediator of the 'wound response' signature, a gene expression pattern associated with poor prognosis in breast cancer (270). Interaction of S100A7 with Jab1 appears to promote the activity of AP-1 and NF-κB transcription factors, as well as resistance to anoikis. Abrogation of Jab1 interaction through mutations of key S100A7 residues causes loss of these effects (266, 271, 272), and reduces the ability of S100A7 to promote cell migration *in vitro* and tumourigenesis *in vivo* (272, 273).

Clinically, S100A7 expression in breast cancer is consistently associated with ER-negativity in both DCIS and invasive lesions (265, 274-276). Additional features associated with S100A7 include increased inflammation (274, 275), high histological grade (265, 275, 276), and lymph node metastasis (274, 276). Consistent with these findings, S100A7+ invasive breast cancers have a poor prognosis (267, 277), and high S100A7 expression in DCIS is associated with progression to invasive disease (266). Of the various relationships between clinical parameters and S100A7, the strongest and most reproducible is that involving ER– status, which is notable given the central importance of ER in breast cancer. Because tumour inflammation and high grade are also frequently associated with ER– status, the association of S100A7 with these factors may actually be due to its relationship with ER. Intriguingly though, the relationship between S100A7 and prognosis is not due solely to ER– status, since S100A7 was associated with poor outcome in a cohort that was entirely ER– (277). This implies that S100A7 expression may denote or contribute to features associated with disease progression for reasons beyond the status of ER and other clinical parameters.

1.7—Summary and key questions

This chapter has described the remarkable biological and clinical heterogeneity of breast cancer. Although this diversity is exploited by some of our most successful targeted therapies (such as endocrine therapy for ER+ tumours), breast cancer heterogeneity also represents a key challenge for clinical management. Tumours that initially respond to targeted therapies frequently recur due to

acquired resistance mechanisms based on either loss of the initial molecular target or development of alternative strategies to support progression. Moreover, many tumours lack molecular targets altogether, leaving non-specific cytotoxic regimens as the only therapeutic recourse. Understanding the mechanisms that underlie tumour evolution and heterogeneity are thus a key challenge for modern breast cancer research.

ER has been stressed thus far as a factor of central importance in breast cancer. This stems both from ER's status as an important therapeutic target and from the fact that ER expression (or lack thereof) is tightly associated with biological subtypes of breast cancer that have inherent differences in their clinical behaviour and prognosis. Given this eminence, it is rather surprising that we lack a clear conceptual framework to explain its presence or absence in breast tumours. One approach to understanding ER expression is to acquire knowledge about the factors with which it associates. In this light, the identification of S100A7 as a tumour-specific and apparently oncogenic factor expressed primarily in ER– disease led us to question how S100A7 comes to be expressed in breast cancer, and whether a common mechanism might also explain the suppression of ER. These two related questions underlie the work presented in Chapters 2 and 3.

Tumour inflammation is rapidly emerging as an important participant in cancer control and progression. During breast cancer development, recruitment of leukocytes is a frequently observed phenomenon that precedes progression from *in situ* carcinoma to overtly life-threatening invasive cancer (9), and experimental models clearly demonstrate that immune responses can be subverted to participate in tumourigenesis. Notably, ER-negativity and S100A7 expression are associated with high levels of inflammation. Indeed, the basal-like breast carcinoma subtype is noted for being almost exclusively ER–, at high risk of metastasis, and for being populated with a robust leukocyte infiltrate. Because there is no clear experimental data to explain the link between inflammation and ER– status (and by association, poor prognosis), discussions of this topic generally boil down to a chicken and egg debate: does the increased tissue destruction caused by aggressive ER– tumours stimulate a more robust immune response, or does the immune system inadvertently provoke the evolution of such tumours? Answering this question would not only satisfy curiosity, but could identify novel therapeutic targets and clarify the cancer-immune system relationship to aid tumour immunologists in devising novel immunotherapeutic strategies.

Ultimately, the process that underlies mortality from breast cancers of all subtypes is metastasis. Given the dismal prognosis associated with metastatic disease, it is essential that we improve our ability to treat metastases or, better yet, prevent them from developing. Two related

concepts that may underlie the metastatic process are EMT and the cancer stem cell hypothesis. In the case of breast cancer, discussions of EMT and stemness lead inevitably to ER and tumour heterogeneity. The same poor prognosis breast cancer subtypes that lack ER expression and harbour dense leukocyte infiltrates are also the subtypes most likely to express genes consistent with a mesenchymal and/or stem cell-like phenotype. Thus, identifying mechanisms of ER regulation may lead naturally to the underpinnings of metastatic behaviour, and vice versa.

This discussion has outlined multiple essential aspects of breast cancer biology that are tightly intertwined. Those relevant to this thesis include breast cancer heterogeneity, ER expression, and the metastatic process. The following chapters will present data relevant to these concepts, beginning with an investigation of mechanisms that underlie S100A7 expression (Chapter 2). In this study, inspired by the effects of inflammatory cytokines on S100A7 expression in the epidermis, we identified OSM as a potent inducer of S100A7 expression in breast cancer cells. Given the link between S100A7 and ER– status, this led to the observation that OSM can exert a similarly potent suppressive effect on ER expression, indicating that OSM may repress the epithelial differentiation of breast cancer cells (Chapter 3). When combined with prior research by others on the pro-invasive effects of OSM, this provided a rationale to explore the impact of OSM on mesenchymal and stem cell-like features in breast cancer, which forms the basis of Chapter 4. While it is tempting to claim that our hypothesis at the outset was "OSM induces the expression of aggressive phenotypes in breast cancer," this would be untrue. Our original hypothesis was that OSM could regulate S100A7 expression, but unexpected observations stemming from this study led us to explore the broader impact of OSM on breast cancer progression. Because each study led naturally to the next, Chapters 2 to 4 are arranged in chronological order to reflect this.

CHAPTER 2

S100A7 (psoriasin) is induced by the proinflammatory cytokines oncostatin-M and interleukin-6 in human breast cancer

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NRW designed the research, performed experiments, collected and analyzed data, and wrote the manuscript. PHW designed the research, analyzed data, and wrote the manuscript.

2.1—Foreword

As described in section 1.6, S100A7 is relevant to both normal biology and disease. Expression of S100A7 under normal conditions appears to be restricted almost entirely to the skin and mucosa, where it serves as an innate defensive molecule that limits bacterial colonization. Beyond these tissues, expression of S100A7 (based on current knowledge) occurs only in the context of pathology. This is certainly the case in mammary tissue, which does not normally express S100A7, but frequently produces it at high levels during neoplastic progression via largely unknown mechanisms.

Multiple aspects of S100A7 biology in breast cancer are worthy of note. Most intriguing is its reproducible and generally strong association with ER– status. Along with the promotion of apoptosis resistance, this is probably a key factor underlying the association of S100A7 with poor prognosis. Its preferential expression in ER– lesions raises the possibility that an improved understanding of its regulation could lead to deeper insights into the biology of ER– breast cancer.

We initially became interested in inflammation as a potential driver of S100A7 expression in breast tumours because of a rapidly growing body of literature in the field of skin inflammation that collectively demonstrated a potent ability of numerous cytokines to induce S100A7 expression in the epidermis. Given the interconnected (but unexplained) associations between S100A7 expression, ER– status, and leukocyte infiltration in breast cancer, inflammatory cytokines within the breast cancer microenvironment were thus attractive candidates to explain the *de novo* expression of S100A7 by malignant breast cells. Cytokine-mediated S100A7 expression in breast cancer is the central concept explored in the remainder of this chapter. This study revealed that inflammatory cytokines, particularly OSM, do indeed regulate S100A7 expression in breast cancer cells, and led us to ask similar questions regarding ER expression that are explored in Chapter 3.

2.2—Abstract

S100A7 promotes aggressive features in breast cancer, although regulation of its expression is poorly understood. Because S100A7 associates with inflammation in skin and breast tissue, we hypothesized that inflammatory cytokines may regulate S100A7 in breast cancer. We therefore examined the effects of several cytokines, amongst which OSM and the related cytokine, IL-6, demonstrated the most significant effects on S100A7 expression in breast tumour cells *in vitro*. Both cytokines consistently induced S100A7 expression in three cell lines (MCF7, T47D, and MDA-MB-468) in a dose- and time-dependent manner. Induction of S100A7 was dependent on STAT3, PI3K, and MAPK signalling, and siRNA-mediated knockdown of S100A7 eliminated the promigratory effects of OSM treatment. *S100A7* mRNA levels in a case-control cohort of human breast tumours (n=20) were significantly associated with expression of the OSM receptor *OSMR* (P=0.0098). This association was confirmed using publicly available microarray data from an independent breast cancer cohort (n=201, P=0.0005) and an association between *S100A7* and poor patient survival was observed specifically in cases with high *OSMR* expression (HR=2.35; P=0.0396; n=85). We conclude that inflammatory cytokines can regulate S100A7 expression and that S100A7 may mediate some of their effects in breast cancer.

2.3—Introduction

A growing body of evidence reveals that the host immune system can paradoxically promote or suppress tumour development depending on the disease context and immune players involved. While adaptive, cell-mediated immune responses can induce tumour regression, many tumours harbour chronically activated infiltrates of innate immune cells and dysfunctional lymphocytes that can support disease progression (149-151). Tumours can use numerous mechanisms to exploit immune activity, including adaptation and responsiveness to immune-derived cytokines. IL-6, for example, a prominent inflammatory cytokine, was recently shown to promote mammosphere formation by putative breast CSCs via Notch signalling (61).

S100A7 (psoriasin) is an inflammation-associated protein relevant to breast tumour progression (267, 278). While generally undetectable in normal breast tissue, S100A7 is frequently expressed at high levels in ductal breast carcinoma, where it is associated with aggressive, high grade, ER– tumours, prominent leukocyte infiltration, and poor patient outcome (274, 275, 277). This is consistent with its stimulatory effects on pro-survival and invasive pathways in breast cancer cells, including PI3K-Akt, NF-κB, and AP-1 (272). S100A7 consistently promotes tumourigenesis *in vivo*

based on two ER– breast cancer xenograft models, in which its expression is either upregulated in MDA-MB-231 cells or reduced in MDA-MB-468 cells (266-268). Although current evidence supports a role for S100A7 in promoting breast cancer, the dominant mechanisms remain to be elucidated. S100A7 expression can be induced *in vitro* by several non-specific stresses such as serum deprivation and high cell density, suggesting that it may be a stress response gene (265). In addition, S100A7 is cooperatively repressed by c-myc and BRCA1 (263) and is positively regulated by ERβ (264). Nevertheless, there is a lack of information regarding well defined environmental cues that transduce S100A7-regulatory signals.

Several groups have identified S100A7 as an epidermal response gene to inflammatory cytokines, including OSM (202, 252). OSM is part of the IL-6 cytokine family, members of which signal through a ubiquitously expressed common receptor chain, gp130, in complex with cytokine-specific secondary chains (e.g. OSMR and IL6R). By virtue of the shared activation of gp130, both OSM and IL-6 trigger similar signalling cascades, involving STATs, MAP kinases, and the PI3K pathway (179).

OSM and IL-6 regulate pleiotropic functions during inflammation. For example, IL-6, along with TNF and IL-1, are products of the classically activated pro-inflammatory macrophage phenotype (156), while OSM may promote neutrophil recruitment to inflamed tissues (199). Both IL-6 and OSM are also implicated in breast cancer progression (238, 279). Several cell types may be responsible for producing these cytokines in tumours. However, while IL-6 is often expressed at high levels by malignant cells (280), in addition to fibroblasts and inflammatory cells, there is little evidence to indicate that OSM is expressed by non-hematopoietic cells (192). Because S100A7 expression is associated with inflammation and promotes aggressive features in breast tumours, we set out to explore the possibility that inflammation may regulate and exert pro-tumourigenic effects through S100A7.

2.4—Materials and methods

2.4.1—Cell culture, transfection, and cytokine stimulation

Human breast carcinoma cell lines MCF7, T47D, ZR75, MDA-MB-231, and MDA-MB-468 (obtained originally from ATCC, Manassas, VA) were cultured in DMEM with 5% foetal bovine serum (FBS) under standard conditions. Characteristic features of cultured lines (morphology, doubling time, etc.) are continually monitored for detection of potential cross-contamination. For stimulation assays, viable cells were seeded into 12-well plates (Corning, Lowell, MA) at 100,000

cells/well (40,000 for long-term withdrawal assays). Human OSM, IL-6, TNF, IL-1β, IL-22, and TGFβ (Peprotech, Rocky Hill, NJ) were stored as 10 μg/ml stocks in culture media. For withdrawal assays, cytokine-spiked media was removed and cells washed twice with sterile phosphate-buffered saline (PBS), followed by continued culture in cytokine-free media. Inhibitors to MEK1/2 (U0126 (281); Cell Signaling, Danvers, MA), PI3K (LY294002 (282)), p38 MAPK (SB203580 (283)), EGFR (AG1478 (284)), or JNK (JNK inhibitor VIII (285); Calbiochem, San Diego, CA) were added (all 10 μM) to cultures 30 minutes before cytokine stimulation. Transient transfection of dominant negative, flag-tagged STAT3 (Y705F; in pRc/CMV; Addgene plasmid 8709 (286)) or empty vector DNA was performed using lipofectamine (Invitrogen, Burlington, ON, Canada) two days before cytokine stimulation. Transfection of STAT3 or S100A7-specific ON-TARGET plus siRNA (Dharmacon, Lafayette, CO) or GFP-specific siRNA (Qiagen, Mississauga, ON, Canada) was conducted using Dharmafect-4 reagent (Dharmacon). Light micrographs were captured using a QICAM camera (QIMAGING, Surrey, BC, Canada) with an Axiovert 40 CFL light microscope (Zeiss, Toronto, ON, Canada).

2.4.2—Western blots

Cells were prepared for western blotting as described previously (274). Protein concentrations were estimated by absorbance at 280 nm using an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE). Primary antibodies were specific for S100A7 (1:1000; Abcam, Cambridge, MA), GAPDH (1:3000; Stem Cell Technologies, Vancouver, BC, Canada), phospho-STAT3 (Tyr705; 1:1000), STAT3 (1:1000), phospho-Akt (Ser473; 1:500), Akt (1:1000), phospho-ERK1/2 MAPK (Thr202/Tyr204; 1:500), ERK1/2 (1:1000), and flag (1:500; Cell Signaling). Secondary antibodies were HRP-conjugated bovine anti-rabbit and goat anti-mouse IgG (1:3000; Santa Cruz Biotechnology, Santa Cruz, CA).

2.4.3—Reverse transcription-polymerase chain reaction (RT-PCR)

Breast tumour samples from primary invasive ductal carcinomas were obtained from the Manitoba Breast Tumour Bank (MBTB), which operates with approval from the Research Ethics Board of the Faculty of Medicine, University of Manitoba. Tissues are accrued to the bank and frozen at –70 °C. A portion of the frozen tissue from each case is processed to create matched formalin-fixed paraffin-embedded and frozen tissue blocks. We constructed a case-control cohort by selecting ten S100A7-positive breast tumours from a larger series of tumours that had been previously assessed

by immunohistochemistry (IHC (277)) performed on the paraffin blocks (and from which frozen tissue blocks were still available), and randomly selected ten S100A7 IHC-negative tumours as controls.

Total RNA was extracted from frozen tissue and cultured cells using the Qiagen RNEasy Mini kit. 1 μg of RNA per sample was reverse-transcribed using MMLV reverse transcriptase as per manufacturer instructions (Invitrogen). Primer sequences and cycling conditions for PCR are provided in Appendix A. PCR products were electrophoresed, stained with ethidium bromide, and imaged using a FluorChem 5500 (Alpha Innotech, San Leandro, CA). Band intensities were assessed using ImageJ. Three independent assays were performed per target, per experiment.

2.4.4—Under-agar migration assay

Migration assays were conducted using six-well plates (Corning) containing semi-solid base-layers of DMEM, 5% serum and 0.5% sterile agar (2.5 ml per well). 1 cm round plugs were removed from base-layers using a bore, producing cylindrical wells within the agar and exposing plate plastic. Equal numbers of viable cells were plated into the agar-wells and cultured for 24 hours. Cells that migrated away from well edges (excluding those still in contact with well edges) were counted in three high power fields (400X) per well and averaged. Each experiment was performed in triplicate.

2.4.5—Statistical analysis

Migration assay results were analyzed using the Student's t-test. Tissue mRNA levels were compared using the Mann-Whitney U-test. *P*-values less than 0.05 were considered statistically significant. All operations were performed using Prism 5.0 (GraphPad, La Jolla, CA).

2.5—Results

2.5.1—Inflammatory cytokines induce S100A7 expression in breast cancer cells

To determine if inflammatory cytokines can regulate S100A7 expression, we treated MCF7 cells for 24 hours with 100 ng/ml of IL-6, TNF, IL-1β, OSM, and IL-22, each of which has been shown to induce S100A7 expression in the epidermis (201-203, 249-254). We observed a robust induction of S100A7 protein following OSM treatment, and a more modest increase in response to IL-6, TNF, and IL-1β (Fig. 3). Despite its role in regulating S100A7 in skin, IL-22 had no apparent effect, nor did TGFβ, which is generally classified as an immunosuppressive cytokine (160). Synergistic effects

on induction of S100A7 were observed when cells were stimulated using either OSM or IL-6 in combination with TNF or IL-1β (Fig. 3). Our subsequent experiments focused on OSM and IL-6, as OSM was the strongest single inducer of S100A7 and IL-6 is in the same cytokine family.

We found S100A7 to be dose-dependently induced by OSM in MCF7 cells, with a minimum effective concentration of 10–15 ng/ml and a maximally effective concentration of 100 ng/ml (Fig. 4a). This effect was also time-dependent at both the protein and mRNA levels (Fig. 4b, 4c). S100A7 mRNA and protein were upregulated by 6 and 24 hours post-stimulation, respectively, and peaked at 48 to 96 hours. Compared to OSM, stimulation with IL-6 caused a weaker induction of S100A7 at late time-points (Fig. 4d). Induction of S100A7 cytosolic protein by OSM was further confirmed by immunofluorescence microscopy (data not shown).

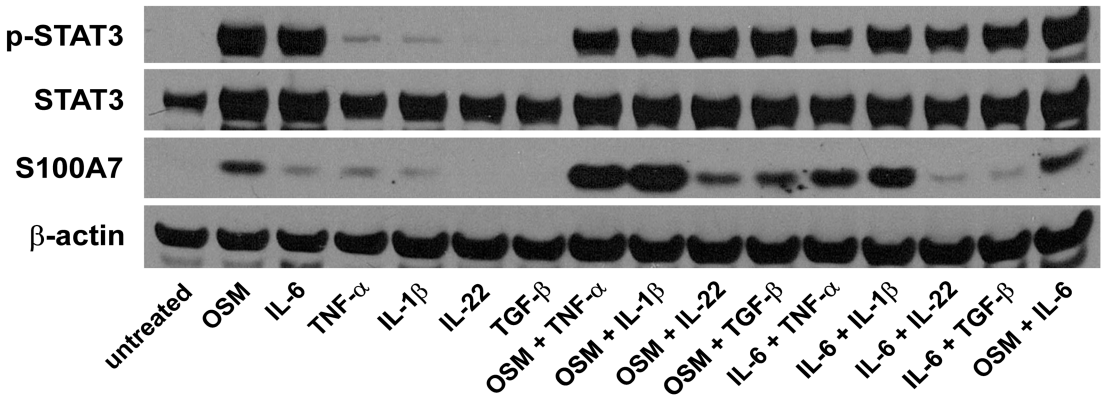

Figure 3. **Inflammatory cytokines induce S100A7 expression in MCF7 cells**. Cells were treated with the indicated cytokines (all at 100 ng/ml) singly or in pairs, for 24 hours before harvesting for western blot analysis. Data are representative of three experiments.

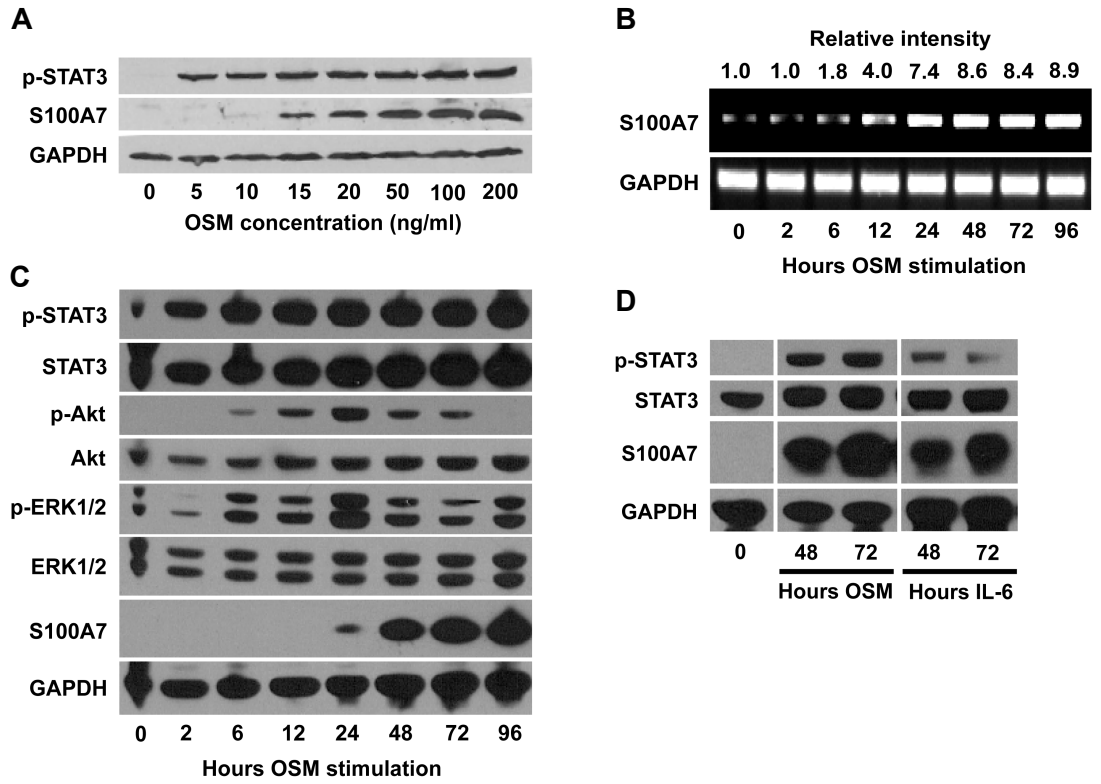

Figure 4. **Dose and time-dependent S100A7 expression following OSM or IL-6 stimulation of MCF7 cells**. (**A**) Dose-dependent induction by OSM (western blot). Cells were treated at indicated concentrations for 96 hours. (**B**) Time-dependent mRNA and (**C**) protein induction by OSM. Media was spiked with OSM (100 ng/ml) at time = 0 and samples collected for western blot or RT-PCR at indicated time points. (**D**) Effect of OSM at late time points on STAT3 activation and S100A7 expression relative to IL-6 (western blot; both cytokines at 100 ng/ml). Data are representative of three experiments.

We next assessed the effects of OSM and IL-6 on the following cell lines: T47D, MDA-MB-231, MDA-MB-468, and ZR75. Stimulation of MDA-MB-231 or ZR75 cells with either cytokine did not affect S100A7 expression (data not shown). However, both T47D and MDA-MB-468 cells responded to OSM and IL-6 by upregulating S100A7 expression (Fig. 5). MDA-MB-468 cells, which express high endogenous levels of S100A7, showed only a modest response to cytokine treatment. In contrast, T47D cells (S100A7-negative under normal growth conditions) responded by robustly inducing S100A7 expression upon stimulation with either OSM or IL-6.

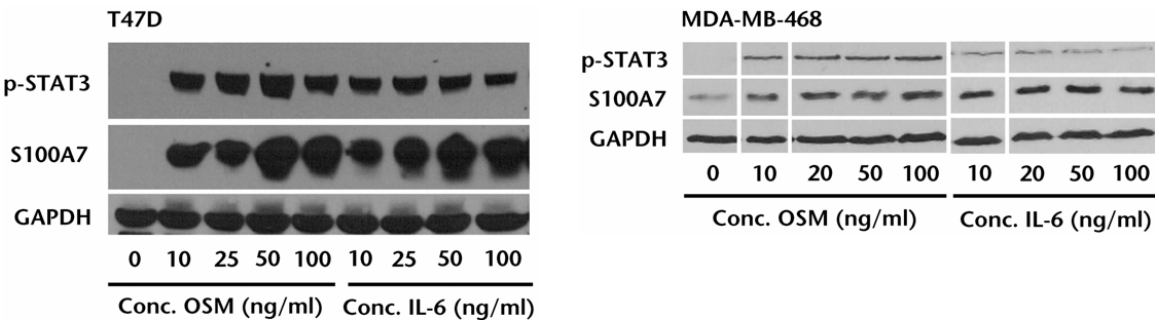

Figure 5. **Dose-dependent S100A7 induction by OSM and IL-6 in MDA-MB-468 and T47D cells**. Cells were treated with indicated cytokine doses for 96 hours before being harvested for western blot analysis. Data are representative of two experiments.

2.5.2—Chronic OSM exposure induces long-term S100A7 expression

Inflammatory cells are a highly variable component of the *in vivo* tumour microenvironment and the concentrations of their cytokine products may fluctuate over time (149-151). To determine if short-term stimulation with OSM or IL-6 is sufficient to induce S100A7, we stimulated MCF7 cells for 2 hours, after which cells were washed with PBS and cultured in cytokine-free media for up to 4 days. In contrast to continuous multi-day treatment, short-term stimulation with OSM was insufficient for full induction of S100A7 expression (Fig. 6a), as was short-term IL-6 treatment (data not shown).

We next examined the long-term persistence of cytokine-induced S100A7 expression. In this experiment, MCF7 cells were treated with OSM for 4 days, followed by up to 3 more days in cytokine-free conditions. Although S100A7 mRNA levels were reduced by 50% after 3 days in cytokine-free media, no reduction in S100A7 protein levels was observed (Fig. 6b, 6c). Similar results were obtained using IL-6 (data not shown). Intriguingly, while levels of activated STAT3, Akt, and ERK1/2 diminished as expected immediately following OSM removal (Fig. 6b), these levels recovered soon after, despite the lack of exogenous stimulation. This suggests that long-term stimulation with OSM triggers a mechanism maintaining activation of signalling pathways that may promote persistent S100A7 expression.

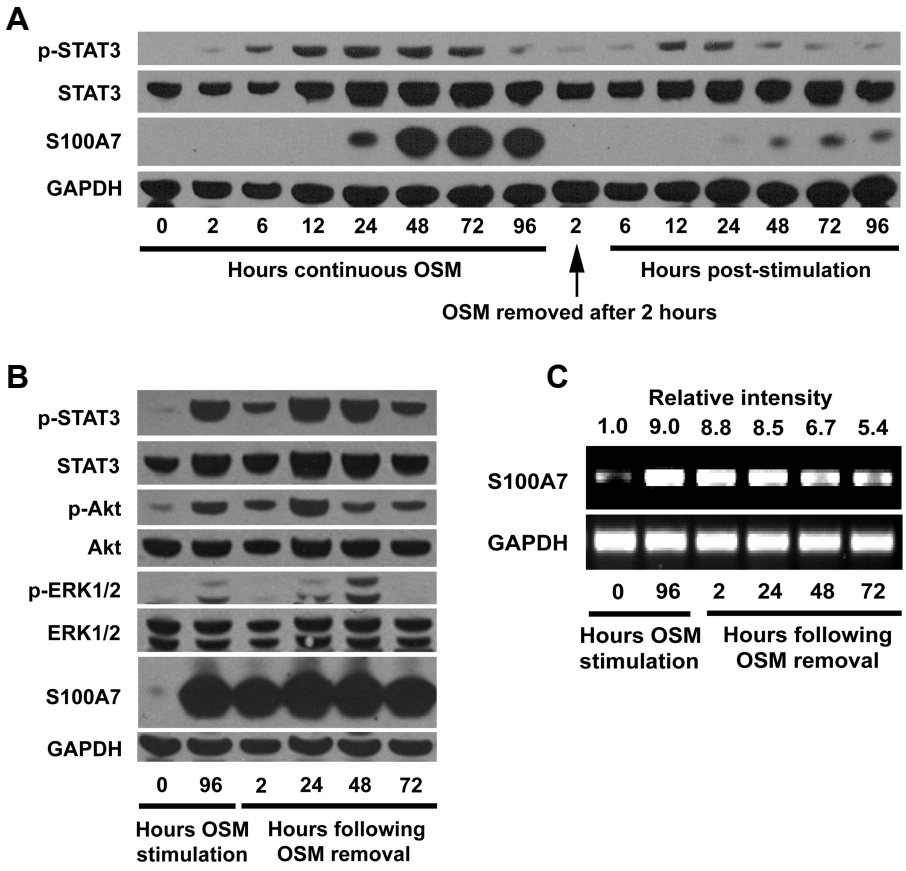

Figure 6. **S100A7 induction in MCF7 cells is related to the duration of the OSM stimulus and can persist for several days following removal of cytokine**. (**A**) Western blot analysis of cells stimulated continuously with OSM over 96 hours or transiently for 2 hours, followed by 96 hours in cytokine-free media. (**B**) OSM stimulation for 96 hours, followed by 72 hours in cytokine-free media (western blot). (**C**) Cells treated as in panel B, but assessed by RT-PCR. Data are representative of three experiments.

2.5.3—OSM induces S100A7 via multiple signalling pathways

OSM and IL-6 are known to activate several signal transduction pathways, including those of STAT3, PI3K, and ERK1/2 (179). OSM additionally activates the stress-activated MAP kinases p38 and JNK (c-jun N-terminal kinase (179)). As expected, STAT3, PI3K, and ERK1/2 were activated over an extended time course in response to OSM stimulation in our experiments (Fig. 4c).

To identify the mechanisms necessary for S100A7 expression, we selectively inhibited each of the above pathways during cytokine treatment. Inhibition of either PI3K (using LY294002) or

MEK1/2 (using U0126) completely abrogated S100A7 induction by OSM and IL-6 (Fig. 7a). Conversely, inhibition of JNK or p38 had no effect (data not shown). Blockade of STAT3 signalling by means of STAT3-specific siRNA or overexpression of a dominant negative STAT3 mutant (Y705F) also inhibited full induction of S100A7 expression (Fig. 7b, 7c). Thus, activation of PI3K, ERK1/2, and STAT3 all appear to be necessary for full S100A7 induction by OSM or IL-6. We (272, 287) and others (268) have previously demonstrated a link between S100A7 and the epidermal growth factor (EGF) pathway. OSM and IL-6 are also functionally related to EGF signalling (184, 234). Intriguingly, inhibition of EGFR using AG1478 caused a marked inhibition of Akt activation and S100A7 expression following OSM treatment (Fig. 8). Furthermore, *EGF* mRNA levels increased during OSM treatment and remained elevated following cytokine removal (Fig. 8). Thus, OSM and IL-6 induce S100A7 in MCF7 cells through a complex mechanism involving PI3K, ERK1/2, STAT3, and potentially autocrine EGF signalling.

2.5.4—S100A7 mediates IL-6 and OSM-dependent migration

A hallmark of OSM-stimulated MCF7 or T47D cells is increased migration and invasiveness, accompanied by loss of cobblestone epithelial morphology (235, 237, 238). To determine if S100A7 is involved in these previously characterized phenotypic changes, we used siRNA to inhibit S100A7 expression during cytokine stimulation in MCF7 cells and assessed cell morphology and migratory capacity. While control transfection with GFP-specific siRNA had little impact on OSM-induced morphology change, it was largely prevented by knockdown of S100A7 (Fig. 9a). Consistent with this effect, siRNA knockdown of S100A7 also abrogated the marked pro-migratory effect of OSM and IL-6 on this cell line, as measured in the under-agar migration assay (Fig. 9b), demonstrating functional relevance of S100A7 in the context of cytokine stimulation. Knockdown of S100A7 by siRNA was verified by western blot analysis (Fig. 9c).

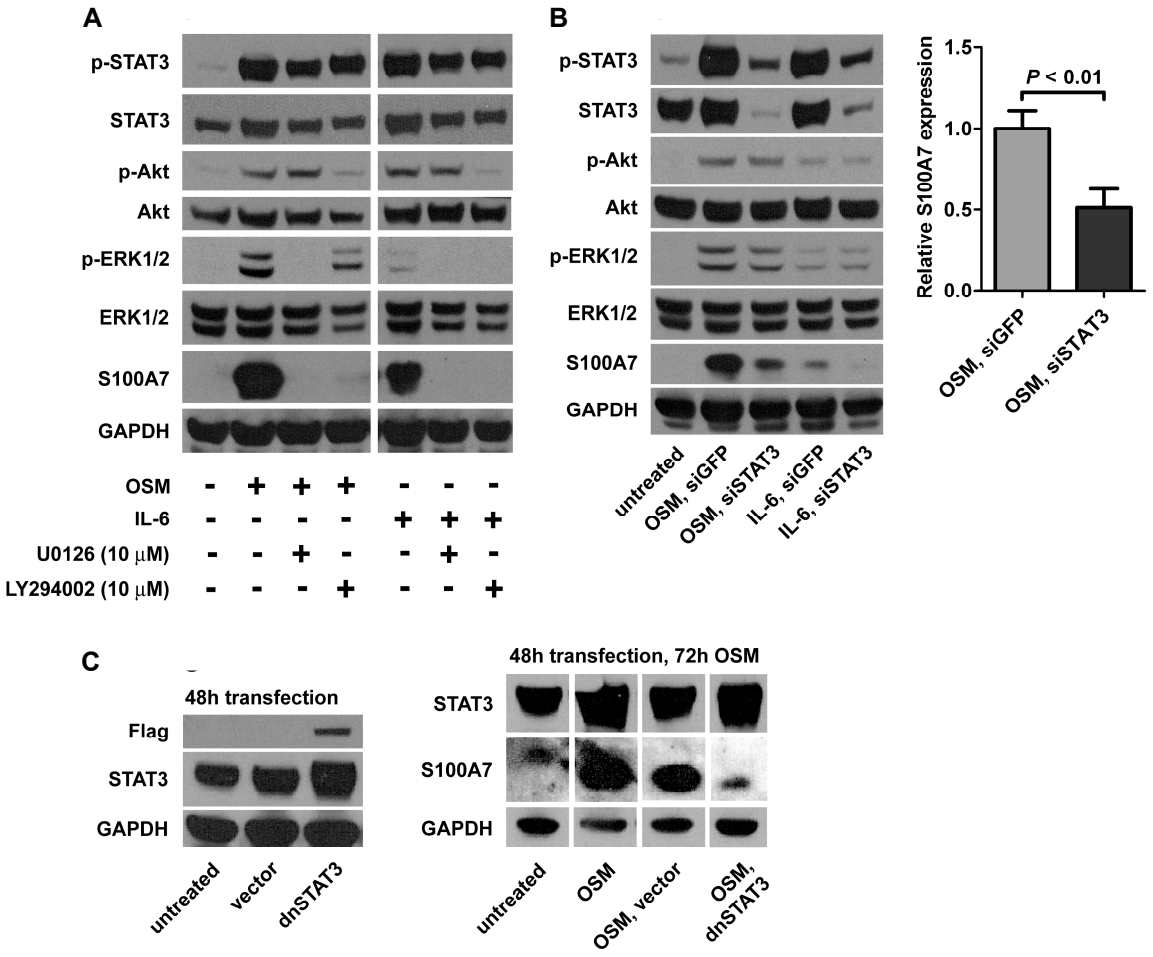

Figure 7. **PI3K, STAT3, and MEK signalling regulate S100A7 in MCF7 cells**. (**A**) OSM/IL-6 stimulation for 24 hours with inhibitors of MEK1/2 (U0126) or PI3K (LY294002). (**B**) STAT3 inhibition by transfection of STAT3 siRNA 24 hours before OSM/IL-6 treatment (left panel). The right panel indicates average (of three experiments, +/- S.D.) S100A7 band densities normalized to GAPDH in OSM-treated cells with or without STAT3 siRNA. (**C**) STAT3 inhibition by transfection with a flag-tagged dominant negative STAT3 (dnSTAT3) mutant (Y705F) 48 hours before OSM stimulation. The left panel indicates successful transfection at the time of OSM treatment. The right panel represents cells harvested after 72 hours of OSM stimulation. All panels depict western blot data and are representative of at least three experiments.

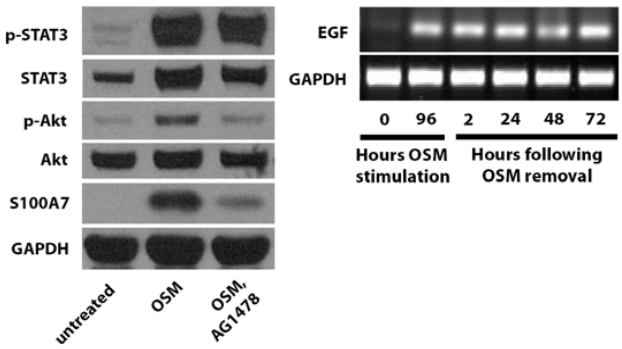

Figure 8. **EGF autocrine signalling may regulate S100A7 expression**. MCF7 cells were stimulated for 48 hours with OSM in the presence or absence of 10 μM AG1478 (EGFR inhibitor; western blot, left panel). In separate experiments, MCF7 cells were treated for 96 hours with OSM, followed by 72 hours of culture in cytokine-free media (right panel). *EGF* mRNA expression was assessed for each time-point by RT-PCR. Data are representative of two experiments.

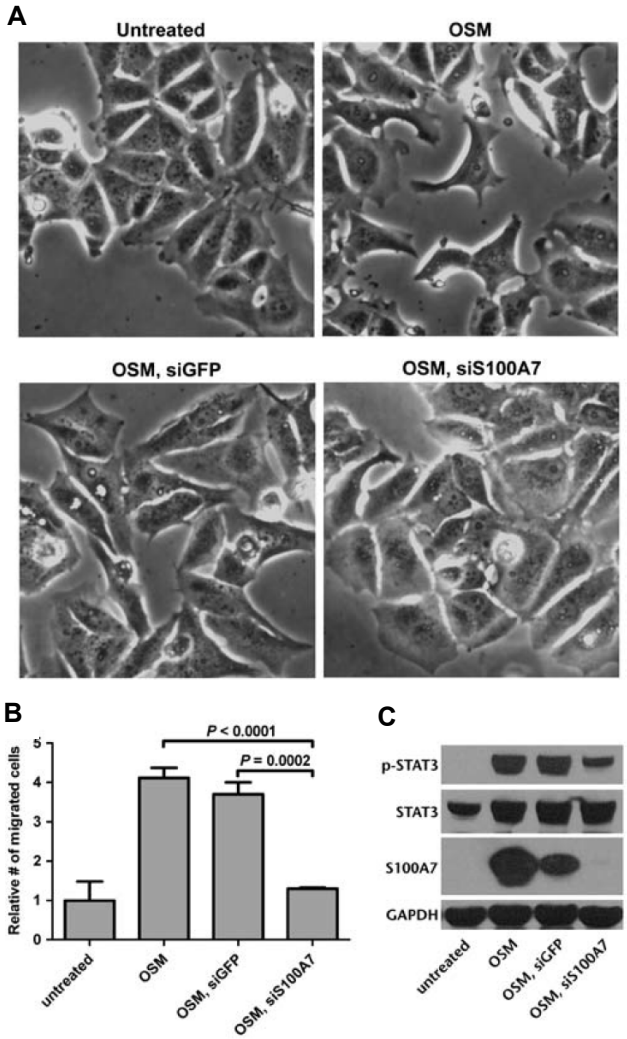

Figure 9. **S100A7 mediates OSM-induced migration in MCF7 cells**. (**A**) OSM induces loss of adhesion, cell spreading, and migratory morphology. Transfection with 100 nM S100A7 siRNA, but not GFP siRNA, 24 hours before treatment prevents this phenotype. Original magnification 400X. (**B**) S100A7 knockdown prevents migration in OSM treated cells, as assessed by the under-agar assay (see Methods). Bars represent mean (+/- S.D) numbers of migrated cells from triplicate samples normalized to the mean of untreated controls, compared using Student's t-test. The data shown is representative of six experiments. (**C**) Western blot verification of S100A7 knockdown by siRNA.

2.5.5—S100A7 is associated with the OSM/IL-6 pathway in vivo

To determine if S100A7 associates with OSM-related genes *in vivo*, we assessed mRNA expression in a cohort of 20 human breast tumours. Using semi-quantitative RT-PCR, we examined the mRNA levels of *OSM*, *OSMR*, *IL6*, *IL6R*, *gp130*, and *LIF* (an additional IL-6 family cytokine) with normalization to *GAPDH* (Fig. 10a). *S100A7* mRNA expression was first assessed to confirm previously determined IHC-based protein detection. We observed 90% concordance (18/20 cases) between IHC and RT-PCR detection of S100A7; the two discordant samples had been previously classified as S100A7-negative by IHC but showed weak *S100A7* mRNA expression by RT-PCR. These samples were reclassified as S100A7-positive for our analyses, leaving a total of 12 *S100A7*-positive and 8 *S100A7*-negative cases. Of the cytokine-related genes listed above, only expression of *OSMR* was significantly enriched (P=0.0098) in *S100A7*-positive tumours (Fig. 10b). However, a secondary analysis in which *S100A7*-positive status was reassigned to the subgroup of tumours with high levels of both S100A7 mRNA and IHC expression (cases 1 to 8) indicated that these tumours were significantly enriched for expression of *OSM* (P=0.041), *IL6R* (P=0.049), and *OSMR* (P=0.023). No significant relationships were evident between *S100A7* and *IL6*, *LIF*, or *gp130*. Because LIFR is an alternative receptor subunit for OSM, we attempted to assess its expression as well. However, while we could readily detect *LIFR* mRNA in positive control tissues (lymph nodes), it was undetectable in any of the breast tumours from this cohort, suggesting that LIFR is expressed at very low levels *in vivo* (data not shown).

To confirm these observations in a larger independent cohort, we obtained publicly available microarray gene expression data from 201 human breast tumours in a previously published dataset (288), accessed via the University of North Carolina (UNC) microarray database. Spearman correlation of median-normalized expression values revealed a strong association between *S100A7* and *OSMR* (P=0.0005) that was retained in the ER– subset (P=0.0013; Table 1). In contrast, *S100A7* was not positively associated with *IL6R*, *LIFR*, *TNFRSF1A* (TNF receptor) or *IL1R1* (IL-1 receptor). Of the cytokines relevant to these receptors, only *OSM* and *LIF* were significantly associated with *S100A7* expression.

If S100A7 mediates aggressive features downstream of cytokine stimulation, this may be reflected in clinical patient outcome. Using the UNC dataset, we sorted cases with associated survival data into those with greater (n=85) or less than (n=78) the median *OSMR* expression level and, within each of these groups, compared the overall survival of patients with high (> 2 times the median value) and low *S100A7* expression (Fig. 11). High *S100A7* expression was significantly

associated with reduced patient survival within the *OSMR*-high population (*HR*=2.35, 95% CI 1.04–5.31; *P*=0.0396), but not the *OSMR*-low population (*HR*=1.04, 95% CI 0.29–3.74; *P*=0.9489). Collectively, these data support the notion that OSM and S100A7 are functionally related in breast cancer, and suggest that S100A7 is a poor prognostic factor primarily in the context of tumours with high levels of *OSMR* expression.

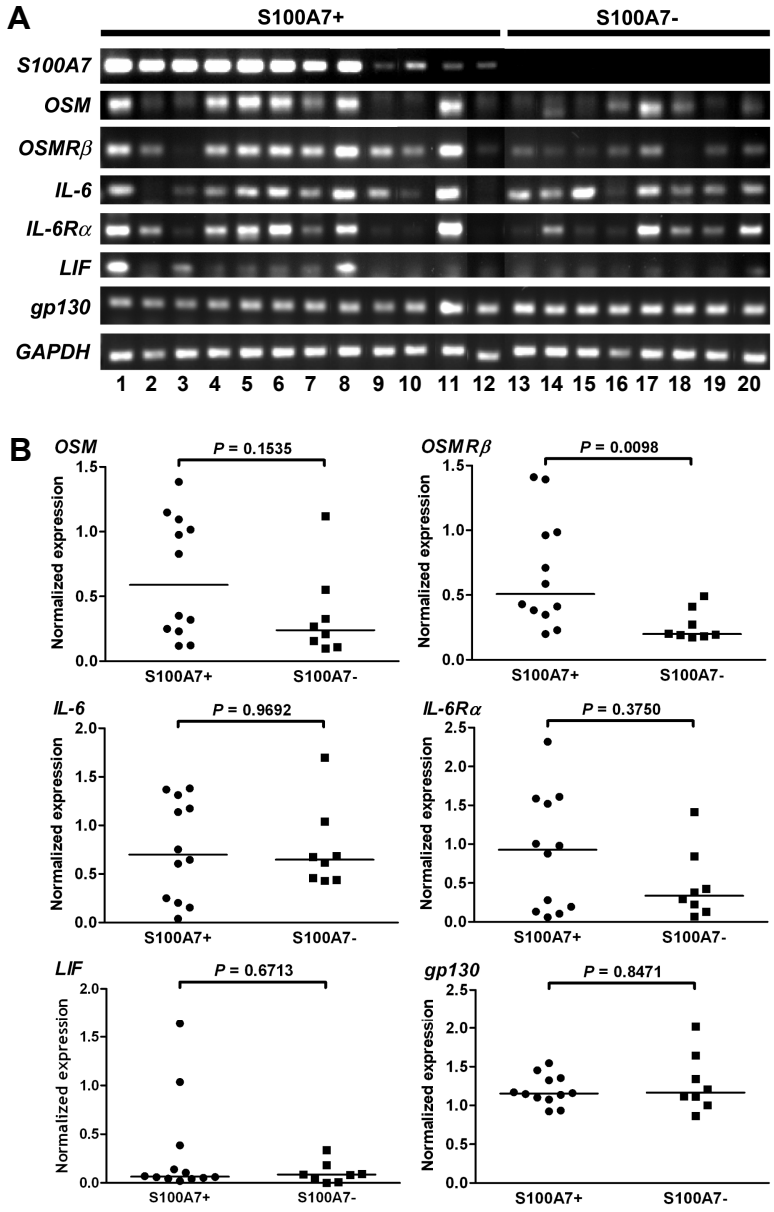

Figure 10. **Association of S100A7 with OSM/IL-6 related genes *in vivo*.** (**A**) Twenty frozen breast tumour samples were chosen based on IHC classification of S100A7 expression. RT-PCR revealed *S100A7* expression in 12 of 20 samples. PCR products of indicated targets from a representative analysis are shown. (**B**) Comparison of gene expression in *S100A7*-positive samples (1–12 in panel A) versus negative controls (samples 13–20). Levels of indicated transcripts were assessed by semi-quantitative RT-PCR. Data points represent *GAPDH*-normalized means of three replicate analyses (bars indicate cohort medians) compared using the Mann-Whitney U-test.

Table 1. Spearman correlation of *S100A7* with cytokine and growth factor-related genes.*

	Total population (*n*=201)		ER-negative population (*n*=84)	
Gene	**r_s value**	***P*-value**	**r_s value**	***P*-value**
OSM	0.1477	0.0373	-0.0333	0.7648
OSMR	0.2446	0.0005	0.3457	0.0013
IL6	-0.0452	0.5259	-0.1215	0.2737
IL6R	0.1300	0.0666	0.1042	0.3454
LIF	0.1639	0.0204	0.3170	0.0033
LIFR	-0.1564	0.0270	-0.0951	0.3896
TNF	0.1034	0.1697	-0.0109	0.9220
TNFRSF1A	0.0397	0.5974	-0.1201	0.2766
IL1B	-0.0455	0.5456	-0.0861	0.4361
IL1R1	-0.0194	0.7968	-0.0101	0.9271
CEBPB	0.2811	<0.0001	0.2931	0.0068
CEBPD	0.1288	0.0691	0.1931	0.0784
EGF	0.1509	0.0330	0.2047	0.0618
EGFR	0.1734	0.0141	0.2264	0.0384
IFNG	-0.0731	0.3034	-0.2493	0.0222

*Data extracted from the University of North Carolina (UNC) microarray database (Herschkowitz *et al*., 2007). Data were expressed as $\log_2$-transformed median-normalized values. The final dataset included 201 specimens of mixed type, including 9 normal tissue samples. ER status was provided in an associated file of clinical data, also accessible within the UNC database (https://genome.unc.edu/). Correlations were performed using Prism 5.0 (GraphPad).

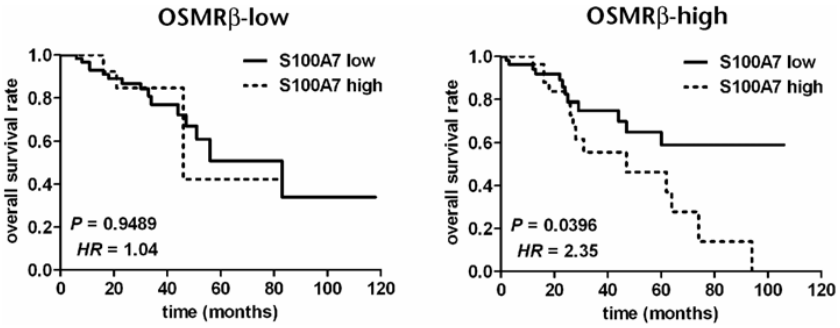

Figure 11. ***S100A7* is associated with poor clinical outcome in an *OSMR*-dependent manner**. Data was extracted from the UNC microarray database (see Table 1 for details of cohort). Patients were sorted into *OSMR*-high and low expression groups. High *S100A7* expression is defined as a $\log_2$ median-normalized expression value greater than 1. Kaplan-Meier curves and log-rank tests were performed using Prism 5.0. HR, hazard ratio.

2.6—Discussion

S100A7 is frequently overexpressed in breast cancer, most commonly in DCIS with a high grade, ER– phenotype (275). The frequent association of inflammation with this tumour phenotype supports the view that S100A7 may be a chemotactic factor for inflammatory cells (256, 257). Alternatively, inflammation may be a causative factor in S100A7 expression. We have shown here that two IL-6 family cytokines, OSM and IL-6, can induce S100A7 expression in breast cancer cell lines via PI3K, MAPK, and STAT3 signalling. Furthermore, we have shown that S100A7 may be a functional mediator of OSM-induced migration. Importantly, a clinically relevant relationship between the OSM pathway and S100A7 appears to exist in human breast cancers *in vivo*. It is notable that while we identified four cytokines capable of inducing S100A7 expression *in vitro* (OSM, IL-6, TNF, and IL-1β; Fig. 3), only the OSM pathway (i.e. OSM and OSMR) was consistently associated with S100A7 levels *in vivo* (Fig. 10; Table 1).

OSM and IL-6 are produced during inflammatory responses by various leukocyte subtypes, including activated T lymphocytes and macrophages (192, 289). Both cytokines exert a variety of effects on breast cancer cells and those of other tumour types. Numerous investigators have examined the role of IL-6 in breast cancer and have shown it to exert both pro- and anti-tumour effects in a context-dependent manner (183). The role of OSM in cancer has been less well studied. Because OSM is cytostatic to breast tumour cells *in vitro*, it was initially thought to have potential therapeutic value. Subsequent studies, however, have revealed that this potential may be limited by the ability of OSM to enhance migration and invasiveness (235, 237, 238). The effects of OSM and IL-6 may contribute to the common observation that chronic inflammation can promote tumourigenesis (149-151). Indeed, we have shown here that OSM may mediate aggressive features via S100A7 expression, and that the OSM/S100A7 axis is associated with reduced patient survival.

Although S100A7 can be highly expressed in breast tumours and is implicated as a gene induced by several forms of cell stress (265, 290), specific mechanisms regulating S100A7 in breast cells have remained elusive. Nevertheless, in non-neoplastic skin pathologies, several investigators have reported S100A7 induction in response to inflammatory cytokines, including OSM, IL-1, IL-17, IL-20, and IL-22 (201, 202, 249, 251-254). Each of these cytokines has the capacity to signal, either directly or indirectly, through STAT3, MAPK, and PI3K (179, 291-293). S100A7 expression in breast tumours was previously shown to correlate with Akt activation (272) and we now demonstrate that OSM and IL-6 can induce S100A7 via PI3K, MAPK, and STAT3. We speculate that this may reflect convergence of all three pathways on the same regulator of S100A7. One

possible candidate may be a member of the C/CAAT enhancer binding protein (C/EBP) family of transcription factors. C/EBPβ and C/EBPδ are both expressed downstream of IL-6 (185). Furthermore, the S100A7 promoter contains multiple C/EBPβ recognition sites and S100A7 correlates strongly with C/EBPβ expression in the UNC microarray dataset (Table 1). In sum, current data suggest that OSM/IL-6 can induce S100A7 through a complex multi-tiered signalling network, a clearer understanding of which is a goal for future study.

In support of the premise that chronic inflammation exacerbates tumour progression, we found that short-term cytokine stimulation of MCF7 cells was insufficient to induce S100A7, suggesting that sustained inflammatory conditions may be required. While STAT3, MAPK, and PI3K mediate S100A7 expression, they may not do so directly, as their activation precedes S100A7 mRNA induction by several hours. Since a direct target of these pathways should become rapidly activated, this implies that one or more additional events take place. This may be related to signals emanating from changes in adhesion or the cytoskeleton, but further exploration of this possibility will be confounded by the observation that S100A7 itself can mediate these changes.

Alternatively, the delay in S100A7 expression may reflect the time required to achieve a critical threshold of signal strength. Indeed, the levels of activated STAT3, ERK1/2 and Akt increase steadily during the first 24 hours of OSM treatment, perhaps as a result of positive feedback (294). Furthermore, as a target gene of NF-κB, IL-6 expression can be triggered by Akt and maintained by subsequent autocrine stimulation (295).

Our data also suggests that high-level induction of S100A7 by OSM may require autocrine EGF signalling. Although our data await confirmation, this is intriguing given prior observations that S100A7 itself may promote EGF expression and is involved in EGFR signalling (268, 272, 287). We have confirmed by analysis of expression levels in an independent breast tumour dataset (UNC) that S100A7 correlates with EGFR expression in a large cohort (and within the ER– subset), supporting the existence of functional cooperativity between S100A7 and EGFR (Table 1).

Once induced by OSM, S100A7 levels appear to remain elevated following cytokine withdrawal. This suggests that, once expressed at high levels *in vivo*, S100A7 expression may persist despite downstream fluctuations in cytokine concentration. From a practical standpoint, this phenomenon may complicate research efforts directed at correlating S100A7 with specific inflammatory mediators *in vivo*. S100A7 mRNA and protein stability is uncharacterized but may be a factor in this persistence. The closely related S100A8 gene exhibits an mRNA half life of approximately 8 hours that can be increased to 20 hours by glucocorticoid treatment (296).

Similarly, S100A4 mRNA and protein half lives are reported to be roughly 8 and 85 hours, respectively (297). However, several other possible mechanisms may underlie this observation. First, autocrine production of IL-6 may continuously drive signals that sustain S100A7 expression. Second, S100A7 itself may establish an autocrine loop, as it is secreted and a putative ligand for RAGE, a promiscuous receptor that signals through NF-κB and MAP kinases (257, 265). Autocrine EGF signalling constitutes another potential mechanism, but is not supported by preliminary evidence (data not shown). Another explanation is based on the role of S100A7 in OSM-induced migration. In MCF7 and T47D cells, fibronectin is upregulated by OSM (235); in multiple myeloma, fibronectin can activate STAT3 in cooperation with IL-6 via β1 integrin signalling (298). If OSM-induced S100A7 causes changes in cell adhesion, this could in turn augment STAT3 activity to help maintain S100A7 expression, thereby perpetuating a loop of pro-migratory signals. The potential S100A7-regulatory mechanisms described above are summarized in Figure 12.

S100A7 is strongly associated with ER– status in breast cancer. Based on our studies of invasive breast cancer, S100A7 is expressed in approximately 50% of ER– versus 20% of ER+ tumours (274, 275, 277, 278). Similar ratios were observed in DCIS, in which 80% of ER– and 30% of ER+ pure DCIS lesions were S100A7 positive (275). We currently show that OSM can induce S100A7 expression in ER+ MCF7 and T47D cells, while the OSM pathway is associated *in vivo* with S100A7-positive tumours that are predominantly ER–. The reason for the association of S100A7 with ER– status *in vivo* is unknown. However, it may be relevant that OSM was shown to reduce ER expression in MCF7 cells (234), an observation that we are actively pursuing. Furthermore, IL-6 correlates with ER– status and poor prognosis in breast cancer patients (183). Therefore, it is possible that OSM/IL-6 signalling may downregulate ER and drive ER+ breast tumours toward an ER– phenotype while concurrently upregulating S100A7, providing a rationale for the link between S100A7 and ER– tumour status.

It was recently reported that treatment of breast cells with IFNγ, a signature cytokine of cell-mediated (Th1) immune responses, suppressed endogenous S100A7 expression (299). OSM and IL-6 differ from IFNγ in that they function more generally as pleiotropic inflammatory signals. Thus, while inflammatory cytokines may positively regulate S100A7, Th1 cytokines could have an opposing effect. This notion is supported by our analysis of microarray data, which reveals a positive correlation between S100A7 and OSMR within ER– tumours, in contrast to a negative correlation with IFNγ (Table 1).

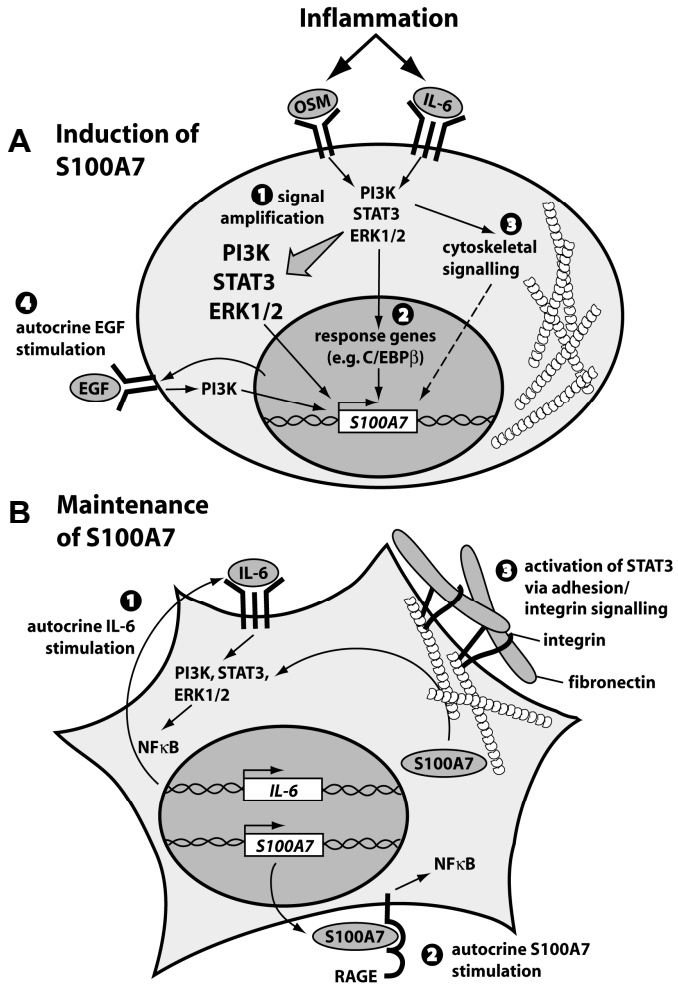

Figure 12. Potential mechanisms of S100A7 regulation by cytokines. (**A**) OSM and/or IL-6 activate STAT3, PI3K, and ERK1/2 which require several hours to induce S100A7, suggesting the necessity of an intermediate step(s). Possibilities include (1) signal amplification, (2) activation of response genes such as C/EBPβ, which in turn regulate S100A7, (3) cytoskeletal signaling, or (4) autocrine EGF signalling. Only options 1 and 4 are directly supported by data in this study. (**B**) Once induced by cytokines, S100A7 expression is independently maintained by an unknown mechanism, for which three possibilities are proposed: (1) autocrine IL-6 signalling maintained by NF-κB, (2) autocrine S100A7 signalling via RAGE ligation, and (3) feedback activation of STAT3 via integrin/cytoskeletal signalling.

While effective anti-tumour adaptive immune responses can be associated with improved clinical outcome, festering intratumoural inflammation is thought to promote malignancy. Indeed, the ability of tumour cells to exploit immune responses for their own benefit by diverse means is increasingly documented (149-151). Expression of S100A7 may represent a novel strategy for malignant cells to translate potentially damaging immune responses into beneficial stimuli. It will be important to achieve a broader understanding of the specific immune parameters regulating S100A7 in the breast, which may reveal additional strategies for targeting S100A7 therapeutically. Furthermore, such knowledge could be relevant in the development of cancer immunotherapies, for which S100A7 expression may be a confounding and potentially damaging side effect of treatment.

In conclusion, S100A7 is a functionally relevant target gene of the inflammatory cytokines OSM and IL-6 in breast cancer. Together with previous data implicating S100A7 in tumour cell survival, these data further support a role for S100A7 in tumour progression. Given the prominence of S100A7 expression in preinvasive DCIS, this also supports the concept that inflammation can influence the earliest stages of breast cancer development through specific modulation of breast cancer gene expression. Further investigation of specific inflammatory conditions regulating S100A7 in breast cancer is warranted, and may reveal novel strategies to target S100A7 therapeutically.

CHAPTER 3

Oncostatin-M suppresses estrogen receptor-α expression and is associated with poor outcome in human breast cancer

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NRW and PHW designed the study and drafted the manuscript. NRW performed the experiments. NRW, LCM, and PHW contributed to manuscript revision and approved the final version.

3.1—Foreword

ER is an essential factor in breast cancer biology. It not only serves as the target for endocrine therapy, but is a vital coordinator of mitogenic signals. ER is also central to breast cancer taxonomy: it is one of three biomarkers used by clinicians to classify breast cancers into clinically relevant subgroups, and is inextricably linked to the various biological 'intrinsic' subtypes defined by gene expression profiling. Because of the limited treatment options for ER– tumours and their inherent aggression, an immense effort to understand their biology and clinical behaviour has been exerted in recent decades. Nevertheless, the question of what dictates the presence or absence of ER expression in breast cancer has (rather bizarrely) rarely been addressed.

Understanding the regulation of ER expression is important for two reasons, the first one being clinical. As a mediator of mitogenic pathways, ER appears to have a dominant effect over other mechanisms of growth and proliferation. For example, restoration of ER expression in ER– breast cancer cells can render them sensitive to the cytostatic effects of endocrine therapies, even though they were fully capable of growth and proliferation in the absence of ER (117). In this light, the ability to target mechanisms of ER suppression to restore ER expression and endocrine sensitivity would be quite useful. The second reason for pursuing mechanisms of ER regulation is related to breast cancer etiology. The evolutionary origins of distinct breast cancer subtypes are a matter of debate. On the one hand, tumours may be ER+ or ER– (or luminal versus basal-like) due to their cell of origin or the specific mutations they acquired during transformation. On the other hand, if breast cancer cells are adaptable and responsive to environmental cues, their ultimate phenotype may be relatively unfixed with little dependence on their cell of origin and underlying mutations. As a central component of breast cancer diversity, elucidating the mechanisms of ER expression would be beneficial for understanding the broader issue of breast cancer etiology.

Our success in demonstrating that inflammatory cytokines (particularly OSM) can induce S100A7 expression in breast cancer led us to ask if they might also regulate ER biology. Beyond S100A7 induction, indirect lines of evidence suggested that this might be true. For example, it has long been recognized that ER– tumours tend to have higher levels of leukocyte infiltration than ER+ lesions. In addition, OSM triggers potent activation of MAPK, a signalling pathway that has been shown by others to regulate ER expression. This chapter provides the first detailed description of ER regulation by OSM, and supports the concept that breast cancer cells can indeed undergo fundamental phenotypic shifts in response to environmental stimuli.

3.2—Abstract

The most important clinical biomarker for breast cancer management is estrogen receptor-α. Tumours that express ER are candidates for endocrine therapy and are biologically less aggressive, while ER– tumours are largely treated with conventional chemotherapy and have a poor prognosis. Despite its significance, the mechanisms regulating ER expression are poorly understood. We hypothesized that the inflammatory cytokine OSM can downregulate ER expression in breast cancer. Recombinant OSM potently suppressed ER protein and mRNA expression *in vitro* in a dose and time-dependent fashion in two human ER+ breast cancer cell lines, MCF7 and T47D. This was dependent on expression of OSMR and could be blocked by inhibition of the MEK1/2 mitogen activated protein kinases. ER loss caused reduced estrogen responsiveness and was also necessary for maximal OSM-induced signal transduction and migratory activity. *In vivo*, high expression of *OSM* and *OSMR* mRNA was associated with reduced ER ($P<0.01$) and progesterone receptor (PR, $P<0.05$) protein levels in a cohort of 70 invasive breast cancers. High *OSM* and *OSMR* mRNA expression was also associated with low expression of *ESR1* (ER, $P<0.0001$) and ER-regulated genes in a previously published breast cancer gene expression dataset ($n=321$ cases). In the latter cohort, high *OSMR* expression was associated with shorter recurrence-free and overall survival in univariate ($P<0.0001$) and multivariate ($P=0.022$) analysis. OSM signalling may be a novel factor causing suppression of ER and disease progression in breast cancer.

3.3—Introduction

ER is a central factor in breast cancer biology. As the primary transcription factor that mediates estrogen signalling, ER is the linchpin of endocrine therapy and a feature that partly defines the various molecular breast cancer subtypes (23, 300). While the mechanisms that regulate ER expression are clearly important for our understanding and clinical management of breast cancer, they remain poorly characterized.

Endocrine therapy is highly effective for the treatment of ER+ breast cancer (70). However, approximately 30% of primary tumours are ER– at clinical presentation, constituting the principal mechanism of intrinsic resistance to endocrine therapy (301). Among tumours that present as ER+ and respond initially to endocrine therapy, some develop acquired resistance through suppression of ER expression, a mechanism that may account for up to 20% of resistant breast cancers (301). Mechanisms to explain ER suppression include hypermethylation of the ER gene promoter (302), destabilization of ER mRNA (303), hypoxia (110), and hyperactivation of MAPK signalling (114,

115, 117, 304, 305). Prolonged growth of breast cancer cells as mammospheres or upregulation of the transcription factors snail and slug may also cause ER suppression (104, 306, 307).

Leukocytes can influence malignant cells through various mechanisms, particularly cytokine release (151), and ER– breast tumours are generally enriched for intratumoural leukocytes compared to ER+ lesions (125). This raises the possibility that tumour-associated immune responses could influence ER expression. Because many of the processes already shown to influence ER are highly dynamic, the ER-negativity of some tumours, whether observed at diagnosis or upon disease progression, might be reversible.

We and others have shown that OSM, a cytokine of the IL-6 family, promotes acquisition of aggressive features such as enhanced migration and invasiveness (235, 237, 238, 308, 309). OSM is produced by leukocytes including T cells, monocytes, and neutrophils (192, 239), and engages heterodimeric receptors involving gp130 and either OSMR or LIFR. Signal transduction (179) is initiated by JAKs that engage the MAPK, STAT3, and PI3K pathways, each of which have documented roles in breast tumour pathogenesis (310-312). Based on the emerging recognition that immune activity can affect breast cancer biology (158), and a prior observation that OSM may influence ER in MCF7 cells (234), our primary aim in this study was to assess OSM signalling as a possible novel regulator of ER expression in breast cancer.

3.4—Materials and methods

3.4.1—Cell culture and cytokine stimulation

Human breast carcinoma cell lines MCF7, T47D, and ZR75-1 (obtained originally from ATCC) were cultured in DMEM with 5% FBS under standard conditions. Human OSM, IL-6, and TNF (Peprotech) were stored as 100 μg/ml stocks in complete culture media and, unless otherwise specified, used at 100 ng/ml.

3.4.2—Chemical inhibitors and RNA interference

Inhibitors to MEK1/2 (U0126 (281) and PD98059 (313); Cell Signaling), Janus kinases (JAK inhibitor I (314)), Ras (FTI277 (315)), JNK (JNK inhibitor VIII (285)), p38 MAPK (SB203580 (283)), PI3K (LY294002 (282)), EGFR (AG1478 (284)), mTOR (rapamycin), or NF-κB (oridonin (316); all from Calbiochem) were added to cultures 30 minutes before cytokine stimulation at doses of 10 μM, with the exception of PD98059 (50 μM), rapamycin (10 nM), and oridonin (10 μg/ml).

Gene knockdown was performed using ON-TARGET plus SMARTpool siRNA at a final concentration of 100 nM, transfected with Dharmafect-4 (Dharmacon).

3.4.3—Western blots

Cells were prepared for immunoblotting as described previously (274). Protein concentrations were estimated using an ND-1000 spectrophotometer (NanoDrop). Primary antibodies were specific for ERα (1:1000; Santa Cruz Biotechnology), GAPDH (1:3000; Stem Cell Technologies), β-actin (1:3000; Abcam) phospho-STAT3 (Tyr705; 1:1000), phospho-Akt (Ser473; 1:500), phospho-ERK1/2 (Thr202/Tyr204; 1:500), and PR (1:500; Cell Signaling). Secondary antibodies were HRP-conjugated bovine anti-rabbit and goat anti-mouse IgG (1:3000; Santa Cruz Biotechnology). STAT3 phosphorylation was used throughout this study as an indicator of OSM functionality (241). Band densitometry was performed using ImageJ.

3.4.4—Real-time quantitative polymerase chain reaction (Q-PCR)

Total RNA was extracted from cell lines and tissues using the Qiagen RNEasy mini kit (Qiagen, Mississauga, ON, Canada) and quantified with an ND-1000 spectrophotometer. RNA was reverse-transcribed using the qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD). Q-PCR was performed using Perfecta SYBR Green supermix (Quanta Biosciences) and an iCycler thermal cycler with a MyIQ real time PCR detection system (Biorad, Hercules, CA). Reactions were performed in triplicate for each sample. Data for all target genes were normalized to *RPL27* expression (317). Intron-spanning primers were purchased from Integrated DNA Technologies (IDT, Coralville, IA) and designed to have annealing temperatures of 60 °C. Primer sequences are as follows (listed 5' to 3'): *RPL27*-forward, CAATCACCTAATGCCCACAAG; *RPL27*-reverse, TTCTTGCCTGTCTTGTATCTCTC; *ESR1*-forward, CGACTATATGTGTCCAGCCAC; *ESR1*-reverse, CCTCTTCGGTCTTTTCGTATCC; *PGR*-forward, TCGCCTTAGAAAGTGCTGTC; *PGR*-reverse, GCTTGGCTTTCATTTGGAACG; *OSM*-forward, CTCGAAAGAGTACCGCG-TG; *OSM*-reverse, TCAGTTTAGGAACATCCAGGC; *OSMR*-forward, TCCCAATACCACA-AGCACAG; *OSMR* reverse, GCAAGTTCCTGAGAGTATCCTG.

3.4.5—ER functional assays

To assay responses to 17β-estradiol (E2, 10 nM), cells were cultured in E2-free media (phenol red-free DMEM with 5% charcoal/dextran-stripped FBS) for 3 days before further treatment. To assess

the effect of ER on cell migration, MCF7 cells were transfected with pcDNA3.1 or pcDNA3.1-ERα using Fugene 6 (Roche, Indianapolis, IN) at a Fugene-DNA ratio of 9 μl to 1 μg. After two days, cells were split into control or OSM treatment groups and one day later seeded in triplicate onto 8 μm-pore polycarbonate filters in 24-well plates at a density of 50,000 cells/well, using FBS as the chemoattractant. Cells were allowed to migrate for 24 hours before they were fixed with 3.7% formaldehyde and stained with crystal violet. Cells on the upper membrane surface were removed with a cotton swab; migrated cells (on the lower surface) were counted in four random fields under high power (200X) and averaged to produce one of three replicate values.

3.4.6—Clinical cohorts

Two independent cohorts of human breast cancer were utilized. The first included 72 invasive breast carcinomas obtained from the MBTB, which operates with approval from the Research Ethics Board of the Faculty of Medicine, University of Manitoba. Cases were selected to represent several molecular subtypes (luminal-A, luminal-B, Her2, and basal-like, as well as triple negative non-basal (TNNB)). ER and PR status were previously determined by ligand binding assay (LBA) and Her2, EGFR, CK5/6, and Ki67 status were determined by immunohistochemistry in previous studies (318, 319). Luminal-A and B tumours were defined as ER+ and/or PR+ (LBA scores of ≥10 and >15, respectively) and Her2– (IHC score <3+); Luminal-B tumours were additionally Ki67+ (>10%+). Her2 tumours had a Her2 IHC clinical score of 3+. Basal-like tumours were triple negative for ER, PR, and Her2, and positive for CK5/6 and/or EGFR (IHC scores derived from the product of intensity and % positivity, as previously described (287)). TNNB tumours were triple negative but lacked CK5/6 and EGFR expression. Frozen tissue sections were used for RNA extraction and Q-PCR. Additional frozen tumour samples not included in the MBTB cohort were obtained from the BC Cancer Agency's Tumour Tissue Repository (REB certificate #H06-60001).

The second cohort was derived from a publically accessible microarray gene expression dataset (Prat *et al*, 2011 (23)), acquired from the University of North Carolina Microarray Database (UNC). Clinical information and intrinsic subtype designations were also acquired from the UNC database. Data from probes matching the same gene were collapsed by averaging. Information regarding assay platforms and individual probes can be obtained from the Gene Expression Omnibus website using accession number GSE18229. Only data from invasive carcinomas were assessed. Data for genes of interest were median-normalized and converted to $\log_2$ ratios before

further analysis. For comparisons among *CD68*-low cases, we renormalized expression values to the *CD68*-low median before analysis.

3.4.7—Statistical analysis

Experiments were repeated at least thrice unless otherwise specified. Tests are specified in figure and table legends; all were two-sided with significance established at $P<0.05$. Univariate and multivariate tests were respectively performed using Prism 5.0 (GraphPad) and Statistics 14 (SPSS, Chicago, IL).

3.5—Results

3.5.1—OSM signalling suppresses ER expression

We began by studying the effect of OSM on three ER+ human breast cancer cell lines. Stimulation of both MCF7 and T47D cells with escalating doses of OSM revealed maximal suppression of ER at a dose of 100 ng/ml after 24 hours of treatment. This corresponded to a reduction in ER protein of up to 95% in MCF7 cells and 85% in T47D cells (Fig. 13a). In contrast, OSM had no apparent effect on ER expression in a third ER+ cell line, ZR75-1 (data not shown). *ESR1* (ER) mRNA levels in MCF7 cells were also significantly reduced after 24 hours of OSM stimulation, as were those of *PGR* (PR), a key target gene of ER transcriptional activity (Fig. 13b). Unlike OSM, stimulation with IL-6, the prototypic cytokine of the OSM family, had comparatively little impact on ER expression (Fig. 13c). Time course assays revealed that although OSM caused rapid (<1 hour) activation of signalling effectors such as STAT3 and ERK1/2, ER protein levels did not become noticeably diminished until after approximately six hours of OSM treatment. With ongoing stimulation, ER levels remained stably suppressed for at least 96 hours (Fig. 13d).

3.5.2—Suppression of ER by OSM depends on expression of OSMR

Because OSM can engage both OSMR-gp130 and LIFR-gp130 receptor heterodimers, we assessed the degree of receptor specificity for the ER-suppressive activity of OSM by knocking down OSMR expression with siRNA. In MCF7 cells transfected with a control GFP-targeted siRNA, 24 hours of OSM treatment caused a four to five-fold increase in *OSMR* mRNA (consistent with prior research (320)), but this was completely abrogated by transfection with OSMR-specific siRNA. OSMR knockdown prevented suppression of both *ESR1* and *PGR* by OSM (Fig. 14a). Blockade of ER suppression was also evident at the protein level (Fig. 14c). As noted above, OSM had a robust

impact on ER levels in MCF7 cells, a reduced effect on T47D cells, and no observable impact on ZR75-1 cells. This is consistent with the level of *OSMR* expression in these cell lines; relative to MCF7 cells, T47D cells have comparable, but lower levels of *OSMR* expression, while expression in ZR75-1 cells is nearly 100-fold lower (Fig. 14b). These data suggest that suppression of ER by OSM occurs principally via the OSMR-gp130 heterodimer.

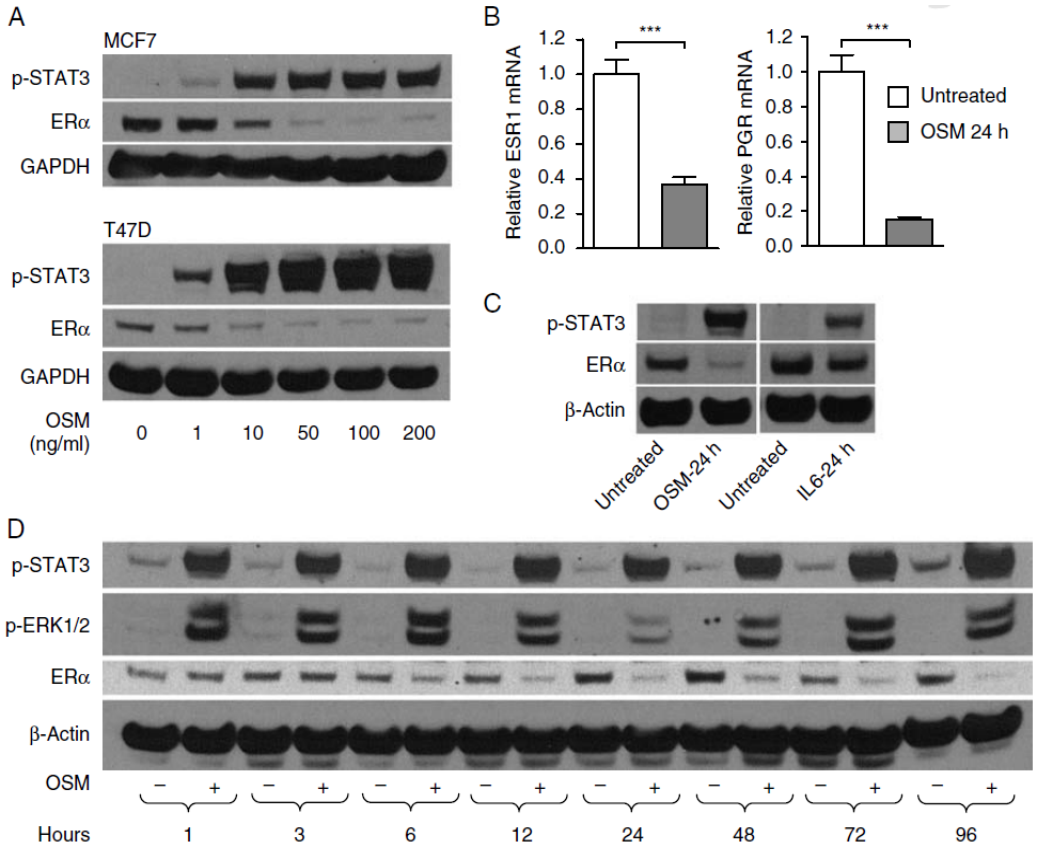


Figure 13. **Suppression of ER expression by OSM**. (**A**) Western blot analysis of MCF7 and T47D cells treated for 24 hours with 1–200 ng/ml of OSM. (**B**) Q-PCR assay for *ESR1* and *PGR* mRNA levels in MCF7 cells after 24 hours of OSM treatment. Bars represent means +/- S.D. ***P<0.001, Student's t-test. (**C**) Western blot comparison of the ER-suppressive effects of OSM versus IL-6 when administered at 100 ng/ml to MCF7 cells. (**D**) Time-course western blot assay of MCF7 cells treated with 100 ng/ml OSM for 1–96 hours.

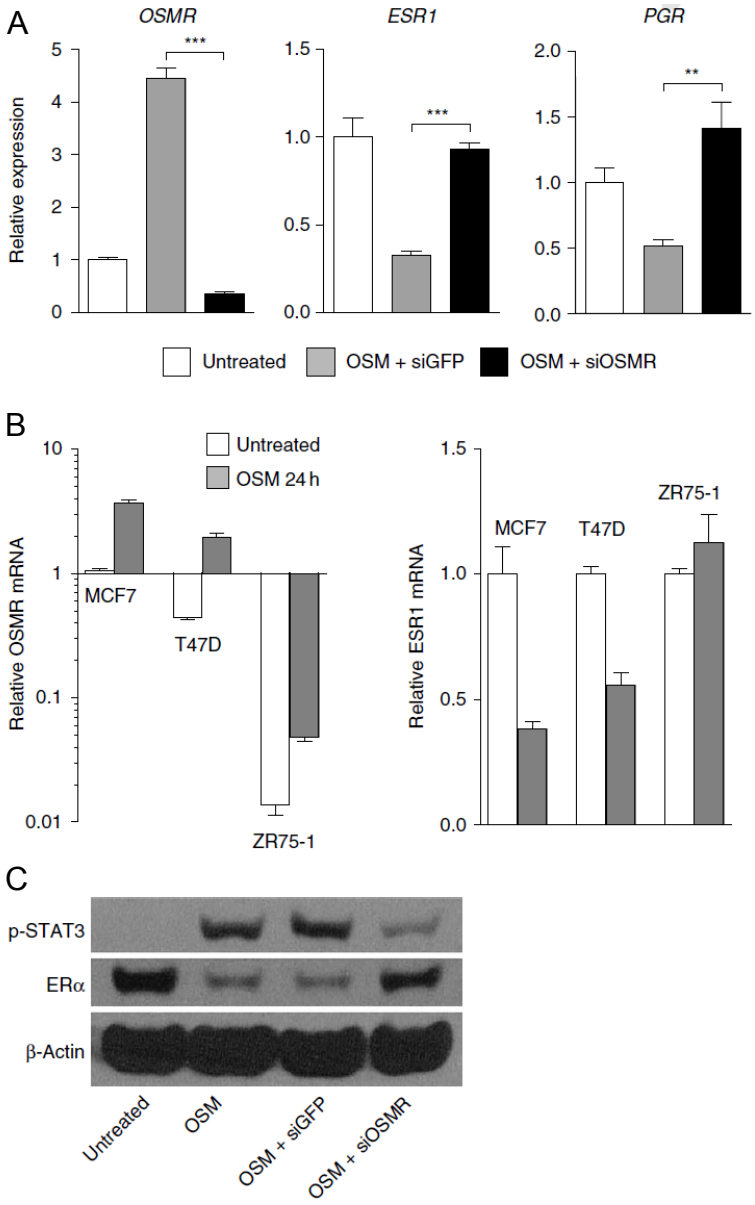


Figure 14. **OSM suppresses ER via the OSMR receptor chain**. (**A**) Transfection of MCF7 cells with OSMR siRNA abrogates the suppressive effects of OSM on *ESR1* and *PGR* determined by Q-PCR. Bars represent means +/- S.D. ***P*=0.001–0.01, ****P*<0.001, Student's t-test. (**B**) Levels of *OSMR* and *ESR1* expression, with and without OSM treatment in three ER+ cell lines: MCF7, T47D and ZR75-1. Levels are expressed as means of triplicate Q-PCR experiments relative to those of untreated MCF7 cells, +/- S.D. (**C**) OSMR siRNA attenuates phosphorylation of STAT3 and loss of ER protein in MCF7 cells.

3.5.3—ER suppression by OSM is reversible and dependent on MAPK signalling

To determine the persistence of ER suppression by OSM, we stimulated MCF7 and T47D cells for 48 hours, withdrew OSM, and cultured the cells for a further 24 hours in cytokine-free media. Withdrawal of OSM caused full restoration of ER expression, indicating that the effect of OSM is transient in the absence of ongoing stimulation (Fig. 15a). To determine the signal transduction requirements for ER suppression, we individually attenuated the STAT3, PI3K, and MAPK pathways prior to OSM treatment. As expected, JAK inhibition blocked downstream signalling pathways and ER loss (Fig. 15b, left panel). Transfection of MCF7 cells with STAT3-specific siRNA failed to attenuate ER suppression (Fig. 15b, right panel), as did blockade of PI3K activity using LY294002 (Fig. 15b, left panel). Treatment with the MEK1/2 inhibitor U0126, however, partially restored ER expression (Fig. 15b, left panel), along with an alternative MEKK inhibitor, PD98059, and the farnesyltransferase inhibitor FTI277, a disruptor of Ras processing and downstream MAPK activity (Fig. 16). Although OSMR physically associates and cooperates with EGFR, inhibition of EGFR with AG1478 did not affect ER, nor did inhibition of the growth factor signalling mediators NF-κB or mTOR (mammalian target of rapamycin) using the NF-κB DNA binding inhibitor oridonin and rapamycin, respectively (Fig. 17). Similarly, although OSM can activate other MAPKs including JNK and p38, inhibition of these pathways had no impact on ER expression (Fig. 17), suggesting that OSM-induced ER suppression may be due specifically to the Ras-MEK1/2 pathway. Intriguingly, blockade of MEK1/2 in MCF7 cells also prevented the morphologic changes characteristic of OSM signalling, implying that ER suppression and the gain of motility-associated morphology may be mechanistically linked (Fig. 15c).

3.5.4—Suppression of ER is functionally important during OSM signalling

To determine if suppression of ER by OSM was functionally relevant, we first assessed the ability of OSM-stimulated cells to respond to the ER ligand, 17β-estradiol (E2). In MCF7 and T47D cells that had been conditioned by growth in hormone free conditions for three days and subsequently stimulated with E2, OSM-treated cells failed to respond to E2 by upregulating expression of PR (Fig. 18a and Fig. 19a). Cell proliferation in response to E2 was similarly inhibited by OSM treatment, though in our experimental conditions this was statistically significant only for T47D cells (Fig. 19b). To assess the role of ER suppression in OSM-induced migration, we constitutively overexpressed ER in MCF7 cells and subjected them to transwell migration assays. Control-transfected cells displayed a six-fold increase in migration 48 hours following OSM treatment. In

contrast, migration was only modestly enhanced by OSM in ER-transfected cells (Fig. 18b), which displayed significantly reduced activation of STAT3 and ERK1/2, but not Akt (Fig. 18c). Collectively, these data indicate that OSM can reduce ER expression below a functionally critical threshold and, furthermore, that suppression of ER may be required for full engagement of OSM-induced signal transduction and cell migration.

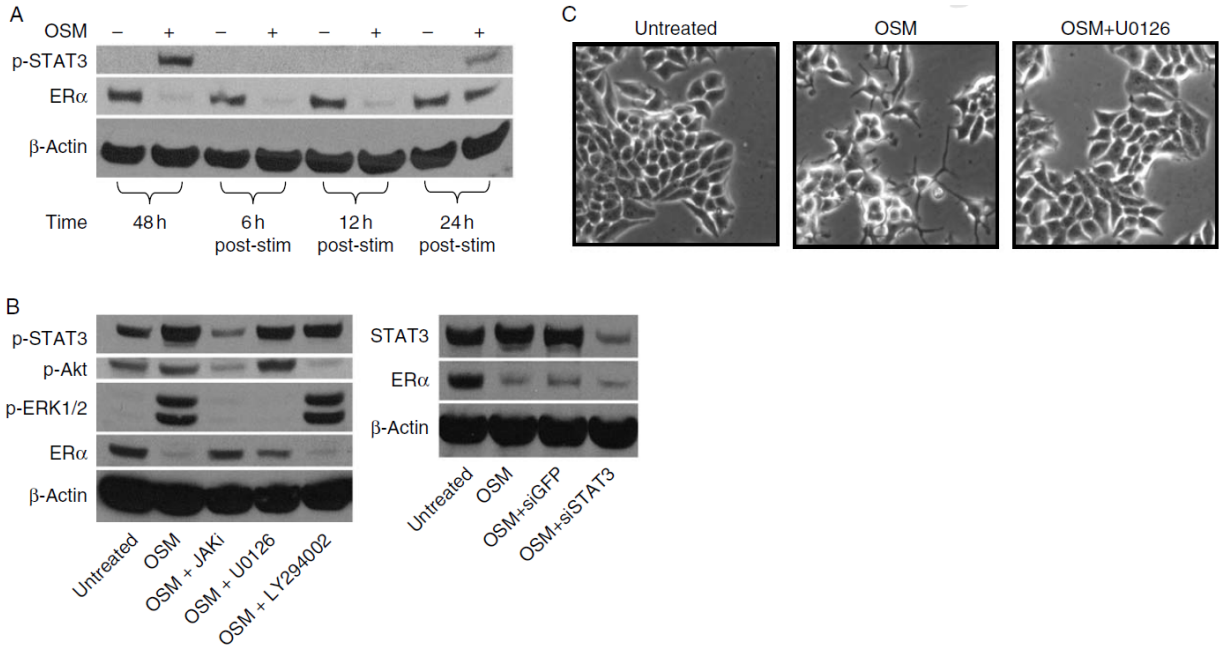


Figure 15. **ER suppression by OSM is reversible and depends on MAPK signalling**. (**A**) Western blot assay of MCF7 cells treated for 48 hours with OSM, followed by removal of cytokine and continued culture for up to 24 hours. (**B**) Western blot analysis of MCF7 cells stimulated with 100 ng/ml OSM in the presence of specific inhibitors of JAK, MEK and PI3K activity (left panel) or STAT3 siRNA (right panel). (**C**) Treatment of MCF7 cells with the MEK1/2 inhibitor U0126 blocks the morphological changes characteristic of OSM signalling. Original magnification 200X.

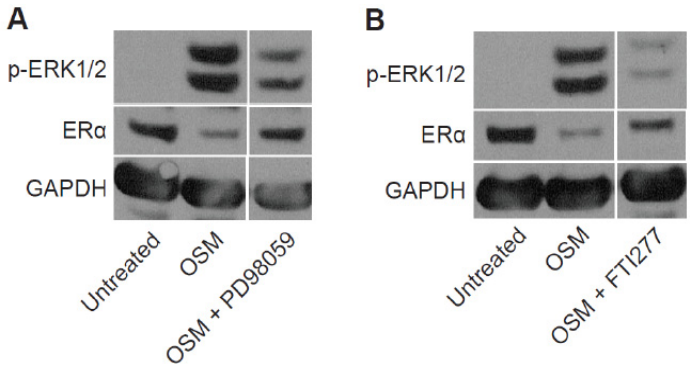


Figure 16. **Blockade of OSM-induced ER suppression using alternative inhibitors of the ERK1/2 MAPK pathway**. Western blot analysis of MCF7 cells demonstrating attenuation of OSM-induced ER loss via blockade of (**A**) MEK signalling (PD98059) and (**B**) Ras activation (FTI277). Experiments were repeated once with similar results.

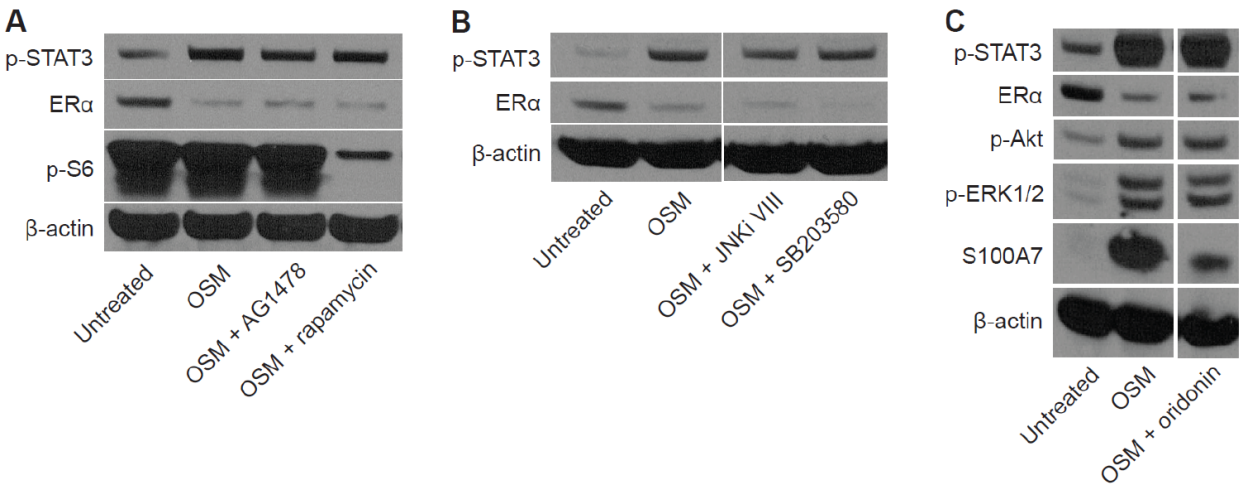


Figure 17. **OSM-induced ER suppression does not depend on EGFR, mTOR, JNK, p38, or NF-κB DNA binding**. Western blot assays of MCF7 cells stimulated with OSM in the presence of inhibitors to (**A**) EGFR (AG1478) and mTOR (rapamycin); (**B**) JNK (JNK ihibitor VIII) and p38 MAPK (SB203580); and (**C**) NF-κB-DNA interaction (oridonin; inhibition of S100A7 induction is included to demonstrate functional efficacy of oridonin). Experiments were repeated at least once with similar results.

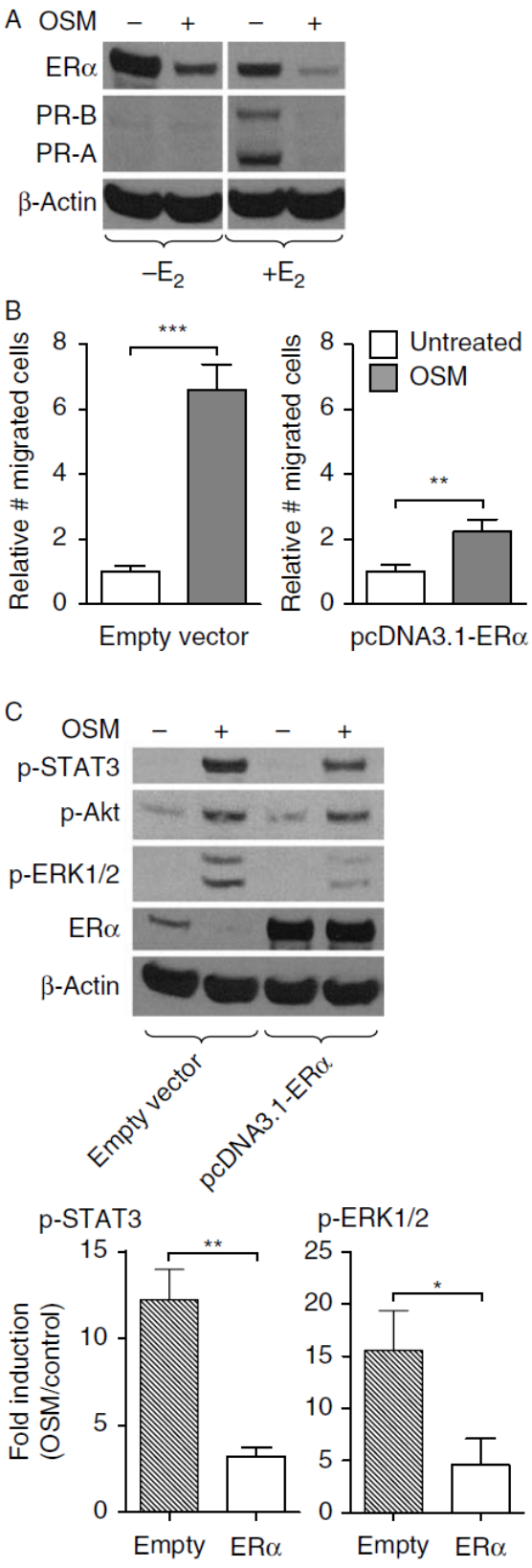


Figure 18. Functional relevance of ER suppression by OSM in MCF7 cells. (**A**) Western blot analysis of PR expression in MCF7 cells after 72 hours of hormone withdrawal, followed by 48 hours of 10 nM 17β-estradiol treatment with or without 100 ng/ml OSM. (**B**) MCF7 cells transfected with empty vector or pcDNA3.1-ER for constitutive ER expression. After 48 hours of transfection, cells were treated with 100 ng/ml OSM for 24 hours and seeded onto 8 μm pore filters in modified Boyden chamber assays. Transmigrated cells were counted 24 hours later. Bars represent the averages (+/- S.D.) of four individual filters, relative to the migration rate of unstimulated cells. ***P*=0.001–0.01, ****P*<0.001, Student's t-test. (**C**, upper panel) Western blot analysis of MCF7 cells treated as in panel B, with corresponding densitometric quantification of p-STAT3 and p-ERK1/2 levels (bottom; expressed as fold induction following OSM treatment in each transfection group). Bars represent mean (+/- S.D.) of triplicate samples. **P*=0.01–0.05, ***P*=0.001–0.01, Student's t-test.

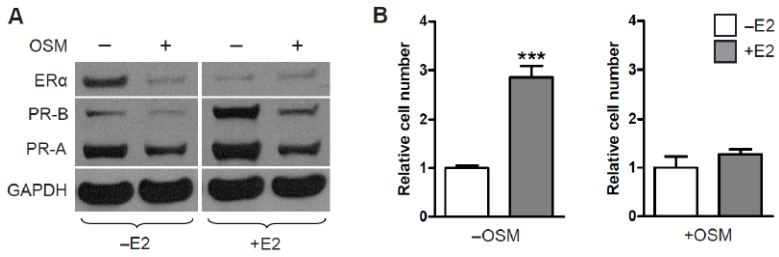


Figure 19. **Functional impact of ER suppression in T47D cells**. (**A**) Western blot analysis of PR expression in T47D treated as in Fig. 18a. (**B**) Proliferation of T47D cells treated as above, relative to E2-unstimulated controls. Bars represent mean (+/- S.D.) relative cell densities after 72 hours of E2-treatment. Cell proliferation was assessed by counting of Hoechst-stained nuclei in triplicate samples. ***P<0.001, Student's t-test. Proliferation assay was repeated once with similar results.

3.5.5—The OSM pathway correlates with suppression of ER in vivo

To investigate the association of OSM signalling with ER in human tumours, we examined *OSM* and *OSMR* expression by Q-PCR in a cohort of 72 invasive breast cancers (MBTB cohort; see Methods—we could not assess *OSMR* in two cases, reducing the effective cohort size to 70). Data were analyzed using median expression values as cutpoints to produce two patient groups: those high in both *OSM* and *OSMR* and those with low levels of one or both. This grouping was chosen on the assumption that both OSM and OSMR must be expressed for full activation of OSM signalling. Overall, 12% (4/32) of ER+ tumours were associated with high *OSM/OSMR* status compared to 45% (17/38) of ER– tumours (Fisher's exact test, P=0.0041) and ER and PR protein levels were reduced by 7–8 fold in the *OSM/OSMR* high group (P<0.05, Fig. 20a). To determine if this association was related to molecular differentiation, we compared the frequency of high *OSM/OSMR* status between each of five molecular subtypes of breast cancer (luminal-A, luminal-B, Her2, basal-like, and TNNB subgroups). High *OSM/OSMR* status was rare in the luminal subtypes (<5% tumours) but was seen in 20–60% of tumours within the other subtypes. When compared to luminal-A tumours, *OSM/OSMR* expression was significantly more frequent within the Her2 and basal-like classes (P=0.0135 and 0.0029 respectively, Fisher's exact test, Fig. 20b). Since high levels of growth factor signalling may independently contribute to suppression of ER (304), a larger subset of Her2+ tumours was included within the cohort to assess the association between *OSM/OSMR* and ER independently of Her2 status. Within this small Her2+ subgroup, there was still a significant inverse correlation between *OSM/OSMR* and ER levels (Spearman r=-0.626, P=0.0014).

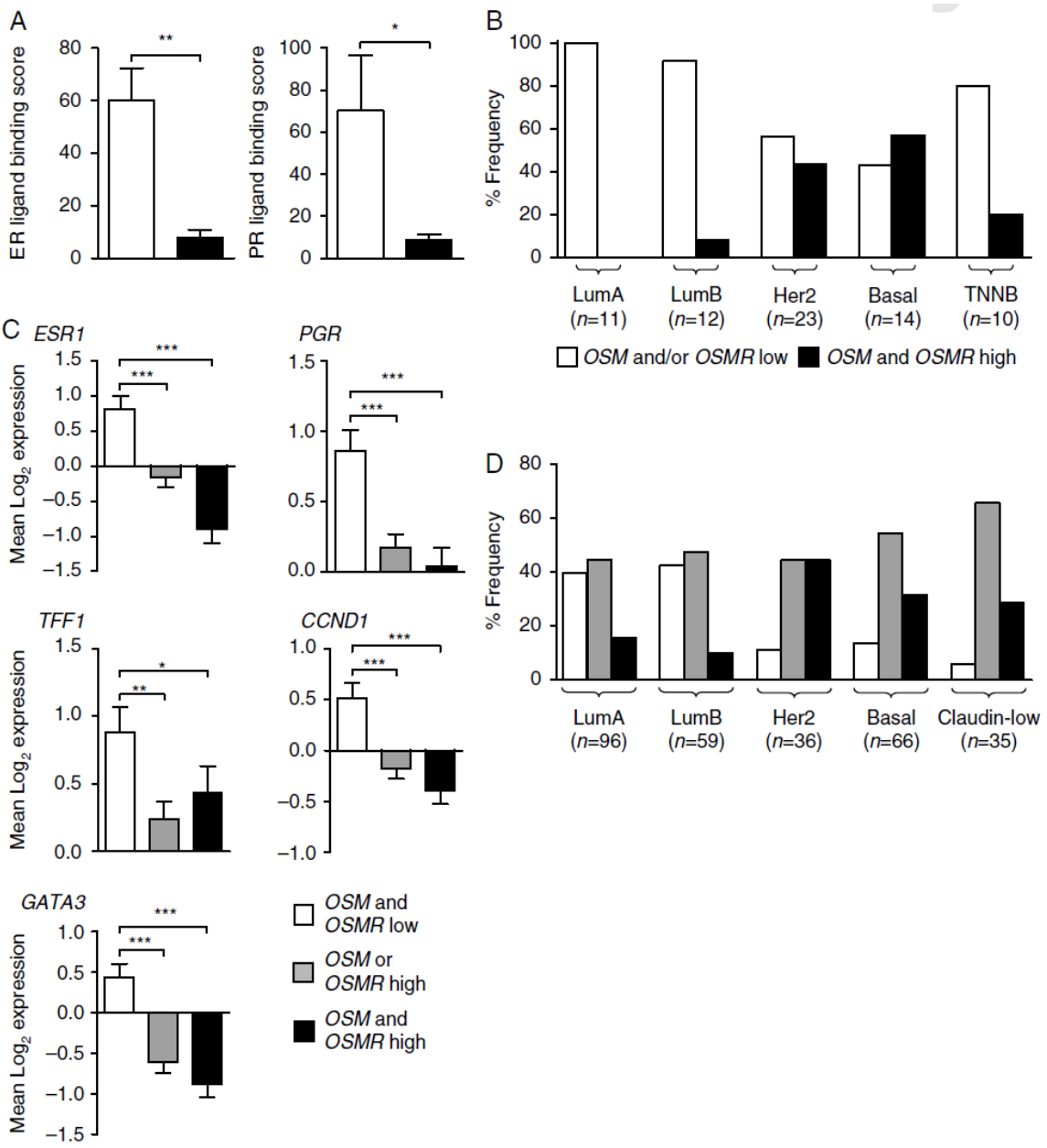


Figure 20. **The OSM pathway is associated with defective ER signalling and aggressive phenotypes *in vivo*.** (**A**) Mean (+/- S.E.M.) ER and PR ligand-binding assay values from 70 breast cancer cases assayed by Q-PCR for expression of *OSM* and *OSMR*. **P*=0.01–0.05, ***P*=0.001–0.01, Mann-Whitney U-test. (**B**) Frequency of high *OSM* and *OSMR* expression in the 70-case cohort, subdivided by inferred molecular subtypes (see Materials and Methods section). Associated legend also applies to panel A. (**C** and **D**) Association of the OSM axis with molecular features in the Prat microarray cohort. (**C**) Association of *OSM/OSMR* expression status with *ESR1* and ER-regulated genes. Bars represent mean expression values +/- S.E.M. **P*=0.01–0.05, ***P*=0.001–0.01, ****P*<0.001, Mann-Whitney U-test. Associated legend also applies to panel D. (**D**) Distribution of *OSM/OSMR* expression across molecular subtypes. In all cases, median expression values were used as the cutpoint to determine high versus low *OSM/OSMR* expression. Molecular subtype designations were previously defined by Prat *et al* (referenced in the main text).

To validate these findings in a larger cohort, we examined data from previously published gene expression microarrays of over 300 invasive breast cancers ('Prat cohort' (23)). Due to the larger sample size of this dataset, we were able to consider three groups of tumours with respect to *OSM/OSMR* expression (using medians as cutpoints): those high in both *OSM* and *OSMR* (high/high, n=77), those high in one or the other (high/low, n=161), and those low in both (low/low, n=83). *ESR1* expression was substantially reduced in the high/low and high/high groups relative to the low/low group, with a clear trend towards lower *ESR1* as representation of the OSM pathway increased (Fig. 20c). This pattern was also largely replicated with respect to four ER-regulated genes: *PGR* (PR), *TFF1* (trefoil factor 1 or pS2), *GATA3* (GATA binding protein 3), and *CCND1* (cyclin D1). This implies that the ER pathway as a whole, rather than simply ER alone, is disabled in tumours with robust OSM activity. Consistent with the MBTB cohort, high *OSM/OSMR* expression was, relative to the luminal-A subtype, strongly associated with the Her2, basal-like, and claudin-low subtypes, each of which are notable for low hormone receptor expression (Fig. 20d; P=0.0003, 0.0007, and 0.0009 respectively, χ^2 test). *OSM/OSMR*-high status was associated with lower *ESR1* expression even when the analysis was restricted to clinically ER– cases (data not shown). As with the MBTB cohort, clinically Her2+ tumours with high *OSM/OSMR* expression had considerably reduced *ESR1* expression relative to low/low cases (P=0.0184, data not shown).

3.5.6—High OSMR expression is associated with poor prognosis

When the prognosis of the *OSM/OSMR* groups described above was assessed in the Prat cohort, we observed increased risk of recurrence in the high/low and high/high groups (P=0.0323; data not shown). However, when *OSM* and *OSMR* were assessed individually, only *OSMR* expression was associated with poor prognosis. To further explore this relationship, we first filtered the cohort by excluding cases with high (upper quartile) expression of the macrophage marker *CD68* (to increase the probability that assessed *OSMR* expression was derived from malignant epithelium, as *OSMR* can be highly expressed in myeloid leukocytes (321)). Cases high in *OSMR* (upper quartile) within this filtered cohort (total n=241) had a much greater risk of recurrence (HR=5.06, 95% CI 2.49–10.29; P<0.0001; Fig. 21a) and overall mortality (HR=5.64, 95% CI 2.63–12.11; P<0.0001). *OSMR*-high status was strongly associated with clinical ER– status (P<0.0001) but not clinical PR or Her2 status, nor lymph node metastasis, patient age, tumour grade, or tumour size (Table 2). In multivariate Cox regression modelling of disease-free survival (DFS) involving the above parameters, *OSMR* was significantly associated with survival (HR=2.75, 95% CI 1.15–6.55; P=0.022)

along with lymph node and PR status (Table 1), and remained significant when molecular subtypes were included in the model (*P*=0.025). Indeed, *OSMR* was strongly prognostic even in the poor outcome basal-like (*P*=0.0087) and Her2 (*P*=0.0001) intrinsic subtypes (Fig. 22). *OSMR*-high status was also associated with loss of coexpression of *ESR1* and ER-regulated genes (*P*<0.0001; Fig. 21b). Among *OSMR*-high cases, those that retained high expression of both *ESR1* and *PGR* had a highly favourable prognosis relative to *ESR1*/*PGR*-suppressed cases (*n*=41, *P*=0.0014; Fig. 21c). This supports the concept that suppression of ER is required for OSM to fully activate an aggressive phenotype in breast cancer cells.

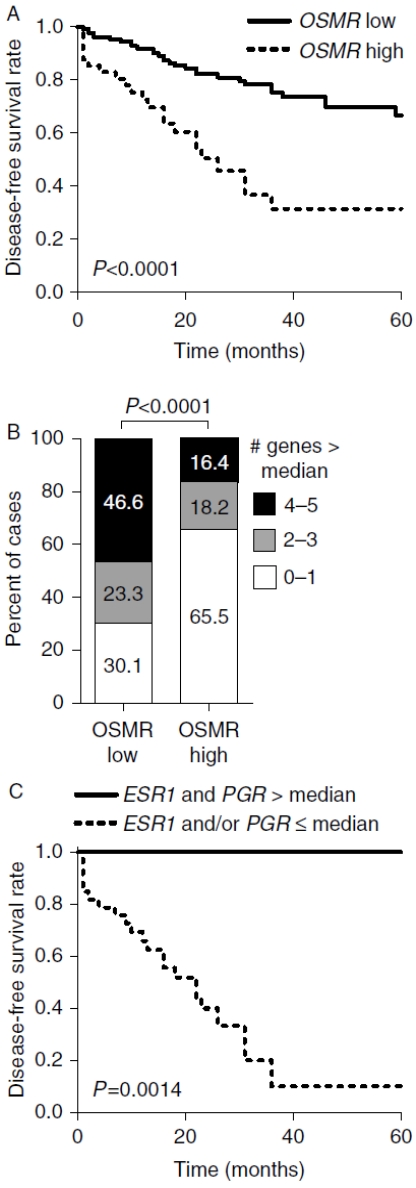


Figure 21. High *OSMR* expression is associated with poor prognosis. Cases in these analyses are those remaining after removal of *CD68*-high cases from the original cohort (see main text). (**A**) Association of *OSMR* with 5-year disease-free survival. *OSMR*-high status is defined as the upper quartile of expression values. (**B**) Expression of ER-regulated genes (*ESR1*, *PGR*, *TFF1*, *CCND1* and *GATA3*) in the *OSMR*-high and -low subgroups. Cases are categorised according to the number of genes with expression values greater than the median. Significance determined by χ^2 test. (**C**) Clinical significance of hormone receptor loss in *OSMR*-high tumours. *OSMR*-high cases are categorised based on retention of both *ESR1* and *PGR* expression (>median) versus loss of one or both. Significance of survival curves was determined by the log-rank test.

Table 2. Prat cohort associations between *OSMR* and clinical parameters and their relationship with DFS in Cox regression modelling.[a]

		Association with ***OSMR***[b]			Cox association with DFS (*n*=132)[c]		
Parameter		***OSMR* low**	***OSMR* high**	***P*-value**	**Comparison**	**HR (95% CI)**	***P*-value**
Patient age	<50 years	53	23	0.0762	<50 vs ≥50	0.48 (0.22–1.05)	0.067
	≥50 years	90	20				
Tumour size	T1	30	9	0.305	T3/4 vs T2 vs T1	1.67 (0.87–3.20)	0.122
	T2	78	18				
	T3–T4	35	15				
Tumour grade	1–2	64	16	0.2848	3 vs 1–2	1.71 (0.72–4.08)	0.226
	3	66	25				
Nodal status	negative	70	14	0.0805	pos. vs neg.	2.85 (1.31–6.20)	0.008
	positive	74	29				
ER status	negative	39	26	<0.0001	pos. vs neg.	1.00 (0.33–3.02)	1.00
	positive	101	16				
PR status	negative	68	22	0.1258	pos. vs neg.	0.28 (0.10–0.84)	0.023
	positive	71	12				
Her2 status	negative	120	34	0.5197	pos. vs neg.	1.84 (0.78–4.31)	0.161
	positive	27	10				
OSMR[d]		182	58	n/a	high vs low	2.75 (1.15–6.55)	0.022

[a]Data reflect the cohort after removal of cases with *CD68* expression within the upper quartile.
[b]Comparisons calculated by two-sided Fisher's exact or χ^2 tests, as appropriate.
[c]Calculated by Cox proportional hazards regression using the enter method.
[d]*OSMR* high cases are those with *OSMR* expression in the upper quartile.

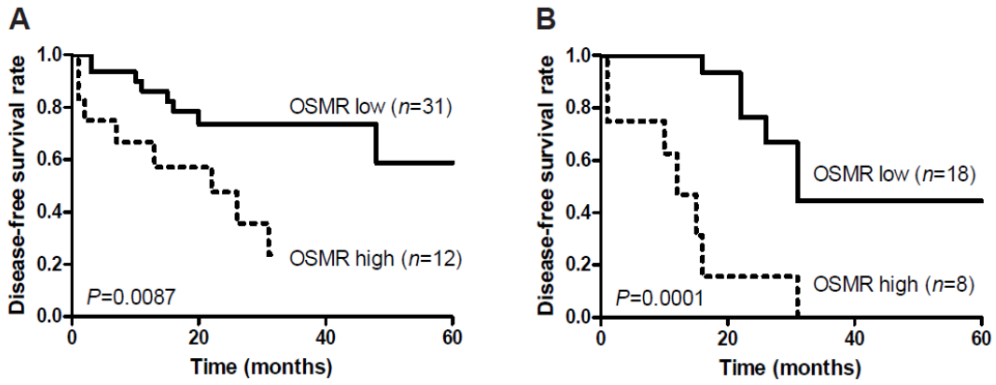


Figure 22. **Prognostic impact of *OSMR* in the basal-like and Her2 tumour subsets**. *OSMR* expression in the Prat cohort with respect to DFS in (**A**) basal-like and (**B**) Her2 intrinsic tumour subtypes. For each comparison, *OSMR* expression was renormalized according to the relevant group median and patients were divided into high and low groups using the upper quartile as the cutpoint. In contrast with Figure 21, all cases regardless of *CD68* status were used for these analyses due to the small number of assessable cases. Significance was determined by log-rank test.

Since the above *in vivo* observations could be attributable to enrichment of *OSM/OSMR* expression in the principally ER– basal-like, Her2, and claudin-low subtypes, we examined luminal-subtype cases within the Prat cohort (>90% ER+) and observed relatively high levels of co-expression of *OSM* and *OSMR* to be associated with reductions in both *ESR1* (*P*=0.0022) and *GATA3* (*P*=0.0009) expression (Fig. 23), two key contributors to the luminal subtype definition (102). High *OSM/OSMR* status was also associated with poor prognosis (*P*<0.0001; Fig. 23) and these observations were not due to enrichment of *OSM/OSMR* expression in the luminal-B subtype.

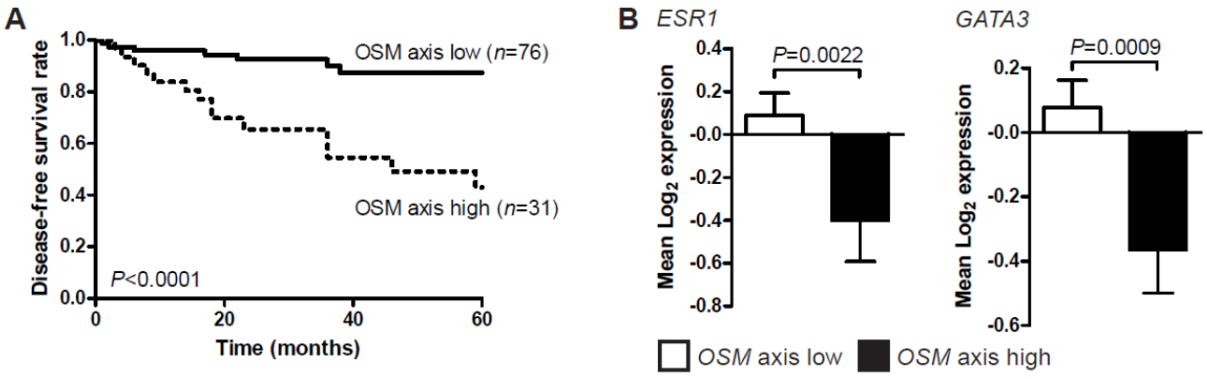
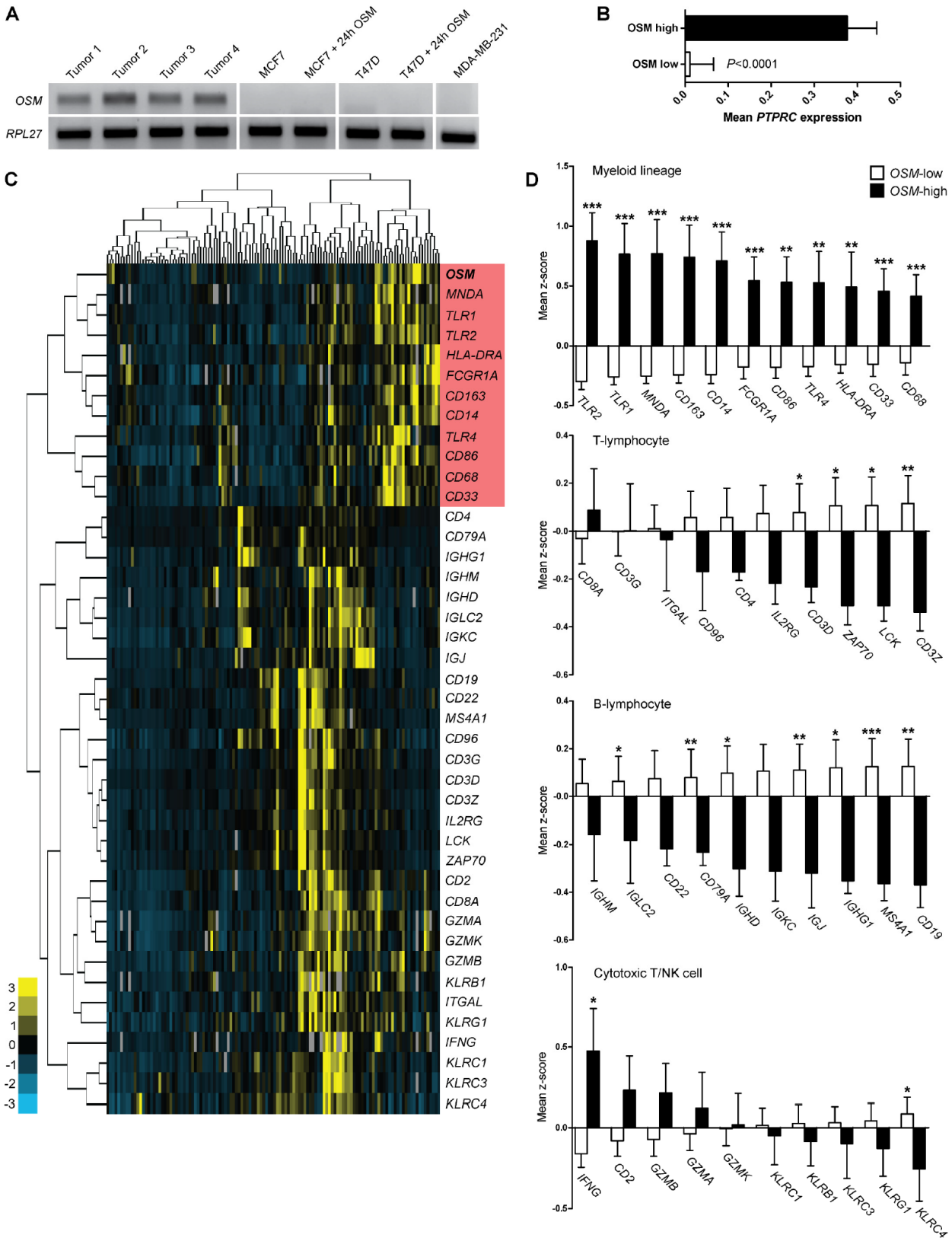


Figure 23. **Clinical relevance of the OSM axis in luminal tumours**. Luminal A and B cases (*CD68*-filtered, as in Figure 21) in the Prat cohort were selected and gene expression data renormalized according to the luminal A/B median. (**A**) Cases were categorized into OSM axis high (*OSM* and *OSMR* > median) and low groups for survival analysis. (**B**) The two outcome groups in panel A were assessed for expression of *ESR1* and *GATA3*, two key genes of the luminal intrinsic subtypes. Statistical significance in panels A and B was determined using the log-rank and Mann-Whitney U-tests, respectively.

3.5.7—OSM expression in breast cancer is associated with innate leukocytes

Although OSM is considered a product of leukocytes, direct evidence for this in breast cancer is lacking. To begin to investigate this question, we examined *OSM* expression by Q-PCR in tumour tissues and cell lines. While *OSM* was readily detectable in breast tumour tissues, we did not detect expression in three breast cancer cell lines (ER+ MCF7 and T47D cells and ER– MDA-MB-231 cells (Fig. 24a)). In the Prat cohort we observed a significant association (P<0.0001) between *OSM* and expression of the pan-leukocyte marker *PTPRC* (CD45, Fig. 24b). Hierarchical clustering of cases with high expression of the immune cell markers *CD247* (CD3ζ) and/or *CD68* (upper quartile, n=122) using genes definitive for distinct leukocyte subtypes yielded expected gene clusters representing myeloid/antigen presenting cells (APC), T cells, B cells, and cytotoxic cells. Of these, *OSM* grouped clearly with the APC subset (Fig. 24c). Analysis of individual genes showed strong associations between *OSM* and APC markers such as toll-like receptors, *CD14*, and *CD163*, but weak, non-existent, or inverse relationships with lymphocyte markers (Fig. 24d). Thus, although this remains to be proven, innate leukocytes are a probable source of OSM in breast tumours.

Figure 24. **Association of *OSM* with leukocytes *in vivo***. *On following page.*
(**A**) Quantitative RT-PCR for detection of OSM was performed using cDNA derived from four frozen ductal breast carcinoma specimens (not included in the MBTB cohort described in the main manuscript), MCF7 and T47D cells with or without one day of 100 ng/ml OSM treatment, and untreated MDA-MB-231 cells (an ER-negative breast cancer cell line). Electrophoresis data is shown because *OSM* mRNA was completely undetectable in cell line cDNA—quantitative assessment of the housekeeping gene *RPL27* demonstrated that this was not due to greater input levels in the tissue samples. (**B**) Cases in the Prat cohort were divided into *OSM* high (upper quartile) and low groups and assessed for *PTPRC* (CD45, in $\log_2$ units) expression to test the association of *OSM* with leukocyte infiltration. (**C**) Cases with high (upper quartile) levels of either *CD247* (CD3ζ), *CD68*, or both were selected, z-normalized, and analyzed by hierarchical clustering using complete linkage and euclidean distance as the similarity metric. The indicated genes were chosen to represent key leukocyte subsets, namely, T cells, B cells, cytotoxic cells (T cells or natural killer cells), and cells of the myeloid lineage. *OSM* clustered together with markers of the myeloid lineage (highlighted) that formed, as expected, a distinct group from the genes representing lymphocytes. Cases in (**C**) are evaluated in (**D**) for associations between *OSM* ('high' refers to the upper quartile) and the individual genes used for clustering analysis, categorized broadly by the expected cell of origin. Bars represent means +/- S.E.M. *P=0.01–0.05, **P=0.001–0.01, ***P<0.001, Mann-Whitney U-test.


A
Tumor 1
Tumor 2
Tumor 3
Tumor 4
MCF7
MCF7 + 24h OSM
T47D
T47D + 24h OSM
MDA-MB-231
OSM
RPL27
B
OSM high
OSM low
P<0.0001
Mean PTPRC expression
C
OSM
MNDA
TLR1
TLR2
HLA-DRA
FCGR1A
CD163
CD14
TLR4
CD86
CD68
CD33
CD4
CD79A
IGHG1
IGHM
IGHD
IGLC2
IGKC
IGJ
CD19
CD22
MS4A1
CD96
CD3G
CD3D
CD3Z
IL2RG
LCK
ZAP70
CD2
CD8A
GZMA
GZMK
GZMB
KLRB1
ITGAL
KLRG1
IFNG
KLRC1
KLRC3
KLRC4
D
Myeloid lineage
OSM-low
OSM-high
Mean z-score
T-lymphocyte
B-lymphocyte
Cytotoxic T/NK cell

3.6—Discussion

We have demonstrated that OSM drives the suppression of ER *in vitro* through a MAPK-dependent mechanism in breast cell lines and that this pathway is part of the pro-migratory phenotype stimulated by OSM in breast cancer. In human breast tumours, the OSM pathway is associated with reduced ER activity and poor prognosis. OSM signalling may therefore be a novel mechanism underlying ER suppression in breast cancer.

The phenotypic heterogeneity of breast cancer, of which ER expression is a key component, is currently explained by two main hypotheses. The first is a lineage-based model in which specific cells of origin (for example, luminal progenitor cells) become mutated and progress to malignancy along defined paths. The second invokes a stochastic model, whereby tumour cells are phenotypically plastic and adjust their behaviour in response to both mutagenic events and shifting environmental conditions. As an explanation for the evolution of ER– breast cancer, our data fit well with the latter hypothesis. Despite their luminal phenotype, MCF7 and T47D cells rapidly downregulate ER upon stimulation with OSM and adopt features typically associated with ER– disease, such as enhanced migration. Suppression of ER and estrogen responsiveness may explain early observations that OSM reduces proliferation of breast tumour cells *in vitro* (322). Upon withdrawal of OSM, hormone receptor suppression is reversible. These findings suggest that breast tumour cells *in vivo* may respond to external factors such as OSM by adopting a phenotype that, through activation of pathways such as MAPK, PI3K, and STAT3, could afford them key advantages including hormone-independent survival, invasion, and dissemination. Upon cessation of cytokine signalling, these cells could restore expression of ER. Such a model could explain the association between leukocyte infiltration and ER negativity, a phenomenon that is not currently understood (125). Furthermore, such a model would imply that some tumours characterized as weakly ER+ or ER– are in fact tumours in which dynamic cell-extrinsic factors serve to suppress ER expression. Indeed, we observed here that ER+ luminal-type breast tumours enriched for *OSM* and *OSMR* had lower *ESR1* expression and a prognosis similar to that of clinically ER– tumours (Fig. 23). It should be noted that our data do not demonstrate that ER+ intrinsic subtypes can evolve directly into ER– subtypes due to OSM. Rather, because OSM signalling appears to be generally associated with depressed ER activity and poor prognosis in all subtypes, our data should only be interpreted as a demonstration that OSM signalling may be one among several possible mechanisms underlying ER suppression in breast cancer, regardless of intrinsic subtype.

The expression of OSMR varies among breast cancer cell lines and may explain much of the variation in their responsiveness to OSM. Compared to MCF7 cells, T47D cells had roughly half the level of *OSMR* mRNA (with a corresponding 30% reduction in the effect of OSM on ER expression), while ZR75 cells had nearly 100-fold less *OSMR* expression and no observable loss of ER in response to OSM (Fig. 14). Consistent with this notion is the observation that *OSMR* expression in breast tumours was more closely associated with clinical outcome than *OSM*. The reasons for variability in OSMR expression in breast tumours are not clear at this time, but may be related to promoter methylation (227, 228). Alternatively, the ability of OSM to induce OSMR expression (Fig. 14) suggests that varying OSMR levels *in vivo* may reflect local OSM concentrations in the tumour microenvironment. It has also recently been shown that c-myc status serves as a determinant of the net cellular response to OSM in breast cell lines (241).

ER suppression following OSM stimulation was, in our experiments, dependent on Ras-mediated MAPK signalling, with no apparent requirement for STAT3 or PI3K activity. The involvement of MAPK signalling is consistent with data from other studies. For example, MCF7 cells engineered to overexpress EGFR or constitutively active Her2, Raf, or MEK exhibited estrogen-independent growth, suppression of ER (114), and expression of a consistent set of MAPK-regulated genes that could accurately predict ER expression in human tumours (115). Furthermore, ER suppression in this model was reversible and could be counteracted using MAPK inhibitors (117). Our work expands on these studies by identifying a physiologically relevant cytokine that potently activates MAPK-dependent ER suppression.

While various leukocyte subtypes are known to produce OSM, evidence that OSM is produced by tumour cells is inconclusive. A single study of its expression in breast cancer tissue was restricted to immunohistochemistry rather than mRNA expression (323). Our preliminary investigation of this issue indicated that OSM is absent in at least three commonly used breast cancer cell lines, and that its expression *in vivo* correlates strongly with markers of innate leukocytes. Intriguingly, a recent study demonstrated that macrophage-conditioned media could suppress ER expression in MCF7 cells in a MAPK-dependent manner (324). However, the specific factors mediating this observation were not identified. Therefore, OSM produced by tumour infiltrating leukocytes may constitute a novel cell-extrinsic mechanism of ER suppression. Further studies involving *in situ* hybridization for *OSM* mRNA localization or direct analysis of specific cell types in fresh breast tumour tissue will be required to conclusively resolve this issue.

Relative to ER+ lesions, ER– breast tumours are enriched in leukocytes and cytokines (124, 125). Nevertheless, little is known regarding the direct effects of cytokines on ER expression. IL-1, IL-6, and TNF are reported to suppress ER expression (109, 119, 120), but other studies have presented conflicting results (121-123). In our hands, IL-6 had little effect on ER expression (Fig. 13c). Clinically, IL-6 has not emerged as a predictor of response to endocrine therapy (325), nor has it shown great utility as a therapeutic target (326). Preliminary data from our lab suggests that TNF does indeed suppress ER and that it can do so synergistically with OSM (unpublished observations). When we examined the expression of inflammatory cytokines and their receptors (OSM, IL-6, LIF, IL-1, and TNF) in the Prat dataset for associations with ER pathway expression and prognosis (as in Fig. 21), only *OSMR* and the TNF receptor (*TNFRSF1A*) were associated with both parameters, though the *OSMR* relationships were considerably stronger (Table 3). Thus, among inflammatory cytokine pathways, OSMR signalling may have a uniquely potent effect on breast tumour biology, for as yet unknown reasons.

Table 3. Associations of inflammatory pathway genes with disease-free survival and expression of the five-gene ER module in the Prat cohort.[a]

Gene[b]	Disease-free survival HR (95% CI)	*P*-value	ER module[c] χ^2 *P*-value	Direction of association
OSMR	5.06 (2.49–10.29)	<0.0001	<0.0001	negative
OSM	1.14 (0.59–2.21)	0.6869	0.0080	negative
IL6R	1.18 (0.62–2.25)	0.6199	0.1181	N/A
IL6	1.18 (0.59–2.33)	0.6406	0.0526	N/A
LIFR	0.95 (0.48–1.90)	0.8948	0.2626	N/A
LIF	1.05 (0.54–2.02)	0.8967	0.9389	N/A
IL1R1	0.95 (0.51–1.77)	0.8611	0.7571	N/A
IL1A	1.80 (0.88–3.66)	0.1051	0.2311	N/A
IL1B	0.82 (0.43–1.54)	0.5322	0.2365	N/A
TNFRSF1A	2.31 (1.06–5.04)	0.0351	0.0029	negative
TNF	0.65 (0.35–1.21)	0.1744	0.1463	N/A

[a]Cases with expression of *CD68* in the upper quartile were omitted from analysis (see main manuscript and Figure 21 for explanation).
[b]Comparisons represent high expression (upper quartile) versus low expression.
[c]ER module—number of the following genes per case with >median expression: *ESR1*, *PGR*, *TFF1*, *CCND1*, and *GATA3* (as in Figure 21 of the main manuscript).

The association of OSMR with poor prognosis was not mirrored by its ligand, OSM, which may reflect a role for OSMR as the key limiting factor in this system. Alternatively, as a leukocyte product, the putative negative influence of OSM may be difficult to separate from the known beneficial impact of host immunity, particularly within ER– lesions (131, 132). Notably, OSMR serves as a receptor for another cytokine, IL-31, which is produced by activated Th2 cells (321). Thus, IL-31 could constitute an alternative OSMR ligand in tumours. The concept of innate leukocytes producing OSM and inducing aggressive changes in neighbouring tumour cells is consistent with the conventional mindset that these leukocytes are largely responsible for the various deleterious effects of anti-tumour immunity (151).

Future investigation of OSM as an ER modulator should involve *in vivo* pre-clinical models to further address the effect of OSM on tumourigenesis, ER expression, and response to endocrine therapy. If such studies verify that OSM signalling promotes breast tumourigenesis, it is possible that this pathway could be targeted therapeutically. Translation to the clinic will require development of strategies for manipulation of either OSM production from macrophages or OSM reception and signalling through OSMR in tumour cells. Some existing chemotherapies are under investigation for their suppressive effects on myeloid cells (327) and new chemical and liposomal drug delivery systems that target macrophages are in development (328, 329). Development and clinical testing of monoclonal antibody-based therapies targeting IL-6 signalling at both the ligand and receptor level are already well advanced (330), suggesting that disruption of OSM/OSMR signalling may be practical. Several features of OSM and OSMR make them attractive potential targets. First, by engaging multiple signalling cascades that potently influence cell survival and migration, such as the STAT, MAPK, and PI3K pathways (179), blockade of OSM signalling would impinge on each of these mechanisms. Second, because gp130 and OSMR signal cooperatively with EGFR-family receptors, blockade of OSMR could potentially attenuate EGFR/Her2 signalling (234). This is particularly pertinent give the current emphasis on clinical testing of agents that target the EGFR family (331). Third, antibody neutralization of OSM and pharmacological inhibition of OSMR-ligand binding are both feasible therapeutic mechanisms. Finally, because the effects of knocking out OSM and OSMR expression in mice appear mild (177), the side effects of targeting OSM signalling may be minimal. Further exploration of the OSM pathway as a potentially clinically relevant modulator of breast cancer biology is warranted.

CHAPTER 4

Oncostatin-M signalling promotes phenotypic changes associated with mesenchymal and stem cell-like differentiation in breast cancer

4.1—Foreword

Chapters 2 and 3 have revealed that OSM stimulation of breast cancer cells can induce concurrent production and suppression of S100A7 and ER, respectively. Both of these changes may be required for the enhanced migratory ability that is bestowed by OSM. The loss of ER and gain of migratory function can be viewed as a disruption of normal epithelial biology, and is consistent with the increasingly popular concept of EMT. It is notable that the breast cancer subtypes characterized by low ER expression, high levels of aggression, poor differentiation (high histopathological grade), and robust inflammation are also those that appear to feature gene expression patterns suggestive of EMT-like processes. In particular, these include the closely related basal-like and claudin-low intrinsic subtypes, two classes that display enrichment of OSM and OSMR expression. In this light, it was natural for us to ask whether OSM signalling can induce additional changes in breast cancer cells that could be considered part of an EMT-like process, particularly given the common view that EMT may underlie the dissemination stages of metastasis.

Although previously an independent concept to explain tumourigenesis, the cancer stem cell (CSC) hypothesis is now linked in the minds of many to EMT. This is based on several publications within the past five years that have demonstrated a role for EMT-promoting factors in enhancing tumour cell stemness, although it is not clear why gain of mesenchymal features should necessarily entail the adoption of a stem cell-like phenotype. Nevertheless, this link is also supported clinically, since human breast cancers that are enriched for expression of mesenchymal genes also tend to express high levels of stem cell-related factors. Thus, in addition to changes associated with EMT, we also examined the capacity of OSM to induce features consistent with CSCs. This chapter demonstrates that breast cancer cells stimulated with OSM do in fact express some additional mesenchymal qualities, along with hallmark features of mammary CSC biology. As with our studies of S100A7 and ER, these changes appear to occur *in vivo* and are likely to be clinically relevant. Thus, OSM signalling not only provides a novel explanation for S100A7 expression and ER loss in breast

cancer, but may also constitute a previously unknown mechanism of inflammation-induced EMT and CSC augmentation.

4.2—Abstract

Two mechanistically linked concepts that are increasingly cited as explanations for cancer recurrence are EMT and the CSC hypothesis. However, our understanding of the stimuli that induce EMT and cancer stemness is incomplete. We and others have shown that the inflammatory cytokine OSM, a member of the IL-6 family, mediates phenotypic changes consistent with an EMT-like process including enhanced migration and loss of hormone receptors. In this study we have expanded on these observations to address the possibility that OSM is a novel cell-extrinsic driver of EMT and/or stemness. OSM stimulation of the well characterized luminal breast cancer cell lines MCF7 and T47D resulted in enhanced expression of the EMT-inducing transcription factors snail and slug, loss of epithelial morphology, and reduced membranous E-cadherin. Consistent with a CSC-promoting effect, OSM treatment markedly increased mammosphere formation by MCF7 and T47D cells (up to 20-fold; $P<0.01$). Mammospheres stimulated with OSM displayed high mRNA expression of *OSMR*, *S100A7*, and the pluripotency gene *SOX2*. The proportion of cells with a $CD44^{high}$- $CD24^{-/low}$ surface phenotype was similarly increased by OSM, in a manner dependent on PI3K and, to a lesser extent, MAPK signalling. Among 72 human breast tumours assayed by Q-PCR, those with high mRNA expression of *OSM* typically expressed high levels of *SNAI1* ($P<0.001$) and *SOX2* ($P<0.05$).Further assessment of publically available microarray data from two cohorts ($n=278$ and $n=241$) confirmed the relationship between OSM signalling and markers of EMT/CSCs, and revealed OSMR as a potential marker of reduced sensitivity to neoadjuvant chemotherapy. Importantly, the association between poor prognosis and high *OSMR* expression may depend on coexpression of *OSMR* and EMT/CSC markers. Our data collectively demonstrate for the first time that OSM can promote changes consistent with EMT- and CSC-like phenotypes in human breast cancer, and that this relationship may be clinically relevant.

4.3—Introduction

Despite ongoing advancement in breast cancer management, our ability to treat recurrent disease remains unacceptably poor. An improved understanding of the processes that underlie treatment resistance and metastasis is urgently needed. In pursuit of this goal, considerable attention has been devoted to the epithelial-to-mesenchymal transition (EMT), during which cells lose epithelial polarity and gain mesenchymal morphology and associated motility. EMT is thought to underpin the processes of migration and invasion that are central to cellular dissemination during metastasis (45). Associated with EMT is the cancer stem cell (CSC) hypothesis, which postulates that tumours are generated by a small population of self-renewing stem-like cells; these are thought to produce additional progeny with limited proliferative capacity that form the majority of the tumour's mass (45). Putative CSCs often express features associated with progenitor cells of the relevant organ (47). In this model, CSCs are considered the primary agents of disease recurrence due to their increased resistance to apoptosis and their ability to propagate new lesions. Indeed, residual breast cancer cells that survive endocrine or chemotherapy are enriched for mesenchymal and CSC features (58). Increasing evidence indicates that the tumour microenvironment may play a key role in regulating EMT and stem-like characteristics in neoplastic cells and, in so doing, may be vital to disease progression (40, 45, 47, 332, 333).

We and others have shown that OSM, a member of the IL-6 cytokine family produced by leukocytes such as T cells, monocytes, and neutrophils (192), can potently induce migration and invasiveness in human breast cancer models (235, 237, 238, 309). In addition, we have recently demonstrated that OSM suppresses the expression of ER in breast cancer cells (334), while concurrently inducing expression of the breast cancer oncogene S100A7 (which is itself associated with ER– disease (309)). The suppression of ER in response to OSM stimulation can be viewed as the loss of a key feature of luminal epithelial differentiation (20). When coupled with the increased migration and invasiveness that are functional hallmarks of OSM stimulation, these observations hint that OSM signalling may promote an EMT-like process in breast cancer cells. By extension, this also implies that OSM may regulate features of CSCs. Consistent with this premise is our prior observation that the OSM pathway is associated with poor prognosis and aggressive subtypes of disease (basal-like and claudin-low) that are known for high expression of genes associated with EMT and stem cells (23, 334). Intriguingly, such tumours are also frequently enriched for infiltrating leukocytes and cytokines (20, 23, 124, 125, 171, 172). Based on this rationale, we investigated whether additional features of EMT and CSCs are induced by OSM in breast cancer.

4.4—Materials and methods

4.4.1—Cell culture and reagents

MCF7 and T47D cell culture, cytokine stimulation, inhibitor treatment, and siRNA transfection was performed as described in Sections 2.4 and 3.4 of this manuscript.

4.4.2—Western blots

Cells were prepared for immunoblotting as described previously (274). Protein concentrations were estimated using an ND-1000 spectrophotometer (NanoDrop). Primary antibodies detected ERα (1:1000; Santa Cruz Biotechnology), GAPDH (1:3000; Stem Cell Technologies), snail (1:500; Cell Signaling), and E-cadherin (1:1000; Abcam). Secondary antibodies were HRP-conjugated bovine anti-rabbit and goat anti-mouse IgG (1:3000; Santa Cruz Biotechnology).

4.4.3—Real-time quantitative polymerase chain reaction (Q-PCR)

Q-PCR was performed as described in section 3.4.4. Primer sequences for each target transcript are listed in Appendix B.

4.4.4—Immunofluorescence microscopy

Cells were grown on glass coverslips in the presence or absence of OSM for 2 days before washing with PBS and fixation for 30 minutes with 3.7% formaldehyde. Cells were washed and permeabilized with PBS containing 0.2% Triton X-100 (PBST) for 10 minutes before blocking with 1% bovine serum albumin in PBST at room temperature for 1 hour. Cells were incubated overnight at 4 °C with primary antibodies against E-cadherin (1:200, Abcam) and/or β-catenin (1:200, Santa Cruz Biotechnology), followed by washing with PBS and incubation for 30 minutes with Alexa Fluor 488 or 555-conjugated secondary antibodies (1:1000, Invitrogen). Cells were washed with PBS, allowed to dry, and mounted onto glass microscope slides using Prolong Gold antifade reagent containing DAPI (Invitrogen). Images were captured using a BX53 light/fluorescence microscope (Olympus Canada, Richmond Hill, ON) with a Nuance multispectral camera (CRi, Hopkinton, MA). Single channel images were directly overlayed using Photoshop CS3 (Adobe, Ottawa, ON).

4.4.5—Mammosphere culture

MCF7 and T47D cells were cultured with or without OSM for two days prior to harvesting by trypsinization. Cells were passed through 40 μm nylon strainers (BD Biosciences, Mississauga, ON) to remove cell clumps. Cells were washed with PBS to remove residual serum and viable single cells were counted by trypan blue exclusion. 2,500 viable cells were plated per well into 96-well ultra-low adhesion plates (Corning, Lowell, MA) containing a total of 150 μl of mammosphere culture medium, composed as follows (concentrations represent final working levels): serum-free DMEM with 1% methylcellulose (from an original stock of 3% in IMDM; R&D Systems, Minneapolis, MN), 20 ng/ml EGF (Sigma-Aldrich, Oakville, ON), 20 ng/ml bFGF (Peprotech), and 1x insulin-transferrin-selenite (Sigma). Addition of methylcellulose is essential to prevent cell diffusion and formation of aggregates that can be mistaken for clonal colonies. After 10 days of culture, images of wells were captured and colonies with a diameter greater than 70 μm were recorded (for colonies not spherical in shape, the longest dimension was measured). Cells that were pretreated with OSM received further OSM stimulation at a reduced dose (50 ng/ml) for the duration of the assay.

4.4.6—Flow cytometry

Cells were harvested using 1 mM EDTA in PBS and stained for 30 minutes at 4 °C with CD44-FITC and CD24-PE monoclonal antibodies at the manufacturer's recommended concentration (BD Biosciences) in flow cytometry buffer (3% FBS in PBS). OSMR is induced following OSM stimulation, but much of this is present as an intracellular pool (320). We thus detected OSMR by intracellular staining: cells stained for CD44 and CD24 were fixed in 4% paraformaldehyde, followed by permeabilization with 0.1% saponin in flow cytometry buffer, and incubation with an OSMR-APC antibody (clone AN-V2; eBioscience, San Diego, CA) in flow cytometry buffer with 0.01% saponin. Cells were analyzed on a BD FACSCalibur flow cytometer and the resulting data processed using FlowJo 7.6.5 (Tree Star, Ashland, OR). Mean fluorescence intensities (MFI) were calculated as geometric means.

4.4.7—Clinical cohorts

Three cohorts of human breast cancer were utilized in this study. The first was comprised of a total of 72 invasive breast carcinomas that we have described previously (Chapter 3 (334)). The second cohort ('MAQC') is publically accessible and contains microarray-derived gene expression data derived from 278 fine needle aspirate biopsies of invasive breast carcinoma (335). Patients were

treated after biopsy collection with neoadjuvant anthracycline-taxane based chemotherapy, and data on clinical outcomes were subsequently collected. Data for specific genes of interest were acquired from the Gene Expression Omnibus website (accession number GSE20194) and median normalized. For analysis of the ER– subset (n=114), gene expression levels were renormalized to the ER– median values. The third cohort ('Prat') is also publically available and was acquired from the University of North Carolina Microarray Database (23). Data from the *CD68*-low subset of this cohort was analyzed as described (Chapter 3 (334)). Because data in the Prat cohort was derived from samples with appreciable levels of stroma, we were cautious in our assessment of genes such as *CD44*, which are highly expressed by leukocytes in addition to tumour cells. For assessment of PI3K signature scores in the Prat cohort, we extracted all available data for genes that comprised the 100 most significant correlates of PI3K activity in the study by Creighton *et al* (116). These data were averaged within individual cases to yield a mean PI3K signature score. In both microarray datasets, median-normalized data were $\log_2$ transformed before analysis.

4.4.8—Statistical analysis

All experiments were repeated at least thrice unless otherwise specified. Specific tests are specified in figure and table legends, with significance established at $P<0.05$. All tests were performed using Prism 5.0 (GraphPad). In all figures, asterisks denote significance levels as follows: *P=0.01–0.05, **P=0.01–0.001, ***P<0.001.

4.5—Results

4.5.1—OSM induces an EMT-like process

Acquisition of migration is a key functional hallmark of EMT, and we and others have noted that OSM is a potent stimulant of migration in breast cancer cells (235, 237-239, 309, 334). To expand on this observation we examined the mRNA expression of several genes associated with EMT in MCF7 and T47D cells that had been stimulated with OSM for 2 days. These cell lines were chosen because they are well established models of breast cancer with luminal epithelial differentiation. OSM treatment induced expression of the transcription factors snail and slug (*SNAI1* and *SNAI2*) in both cell lines (Fig. 25a). However, OSM did not induce expression of twist (*TWIST1*) or vimentin (*VIM*), both of which are commonly associated with mesenchymal transitions. Fibronectin (*FN1*), *FOXC1*, and *ZEB1* were variably and inconsistently induced between the two cell lines. Intriguingly, although MCF7 cells suppressed E-cadherin (*CDH1*) and increased N-cadherin (*CDH2*) expression,

this was not observed in T47D cells, despite the known pro-invasive effect of OSM on this cell line. The effect of OSM on snail and E-cadherin was confirmed by western blot (Fig. 25b).

Loss of E-cadherin is considered an essential hallmark of EMT in both normal and neoplastic settings (336). The sequestration of β-catenin at the cell membrane is an important component of E-cadherin-based adherens junctions. Under control conditions, immunofluorescence microscopy demonstrated clear membranous colocalization of E-cadherin and β-catenin in both MCF7 and T47D cells, which exhibited characteristic cobblestone morphology (Fig. 25c). Consistent with the observations in Figure 25a, OSM caused a reduction in E-cadherin membrane localization in MCF7 cells with a corresponding intracellular redistribution of β-catenin. Notably, OSM-treated cells that exhibited the greatest degree of intracellular β-catenin expression tended to be detached from cell islands and displayed apparent migratory morphology (Fig. 25c, inset). Despite having no detectable effect on overall E-cadherin expression in T47D cells (by western or Q-PCR analysis), OSM treatment nevertheless caused a reduction in surface E-cadherin expression—many T47D cells displayed intracellular inclusions that stained positive for E-cadherin, suggesting that T47D cells can upregulate migration in response to OSM by internalizing E-cadherin, as opposed to degrading it or blocking its expression (Fig. 25d).

As discussed in Chapter 3, OSM suppresses ER expression in breast cancer cells (334). Because snail overexpression in MCF7 cells has also been shown to suppress ER (104), we blocked OSM-induced snail expression in MCF7 cells by transfection with snail siRNA (Fig. 25e), but observed no change in OSM-mediated ER suppression. Unexpectedly, this was also the case with E-cadherin, suggesting that additional OSM-regulated factors are at play (possibly slug). However, snail knockdown did prevent induction of S100A7, an OSM target gene that mediates migration in this cell line (Chapter 2 (309)), implicating S100A7 as a functional effector of EMT. Because β-catenin is implicated as an inducer of EMT through its stimulatory effect on snail expression (337), we also knocked down β-catenin using siRNA—however, in the context of OSM stimulation, β-catenin knockdown in MCF7 cells caused extensive cell death (data not shown), which prevented us from fully assessing its role in OSM-induced EMT processes.

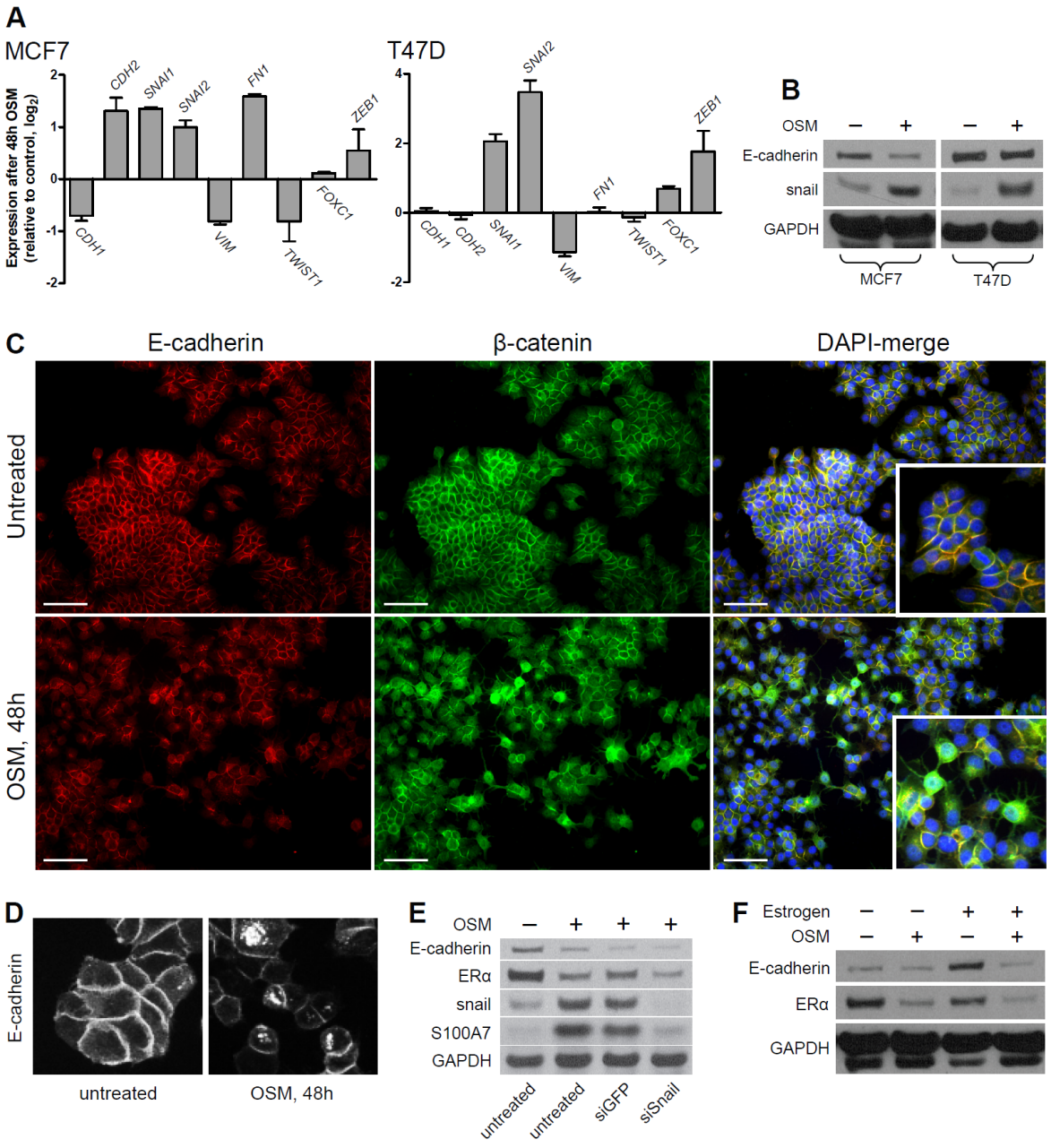


Figure 25. **Induction of EMT features by OSM**. (**A**) Q-PCR assessment of EMT-associated gene expression in MCF7 and T47D cells stimulated with OSM. Bars represent mean (+/- S.D.) expression levels relative to untreated cells. (**B**) Western blot detection of E-cadherin and snail following OSM treatment. (**C**) Immunofluorescent detection of E-cadherin (red) and β-catenin (green) in MCF7 cells. DAPI is included as a nuclear counterstain. Scale bars equal 100 μm. (**D**) Immunofluorescent E-cadherin staining in T47D cells, showing intracellular localization following OSM treatment. (**E**) Western blot analysis of MCF7 cells transfected with GFP (control) or snail-specific siRNA. (**F**) Western blot analysis of MCF7 cells treated with 10 nM 17-β-estradiol and/or OSM for 24 hours following 3 days of estrogen withdrawal (culture in phenol-red free DMEM with 5% charcoal stripped FBS).

Because ER has been shown to augment E-cadherin expression in breast cancer cells (338), we tested the effects of estrogen stimulation on estrogen-starved MCF7 cells in the context of OSM treatment. Consistent with prior findings, estrogen treatment caused an increase in E-cadherin protein levels, but this was abrogated in cells concurrently treated with OSM (Fig. 25f). Thus, loss of ER expression in MCF7 cells following OSM treatment may contribute to OSM-induced E-cadherin repression.

4.5.2—OSM enhances features of breast cancer stem cells

Because gain of mesenchymal features are associated with the induction of a CSC-like phenotype, we examined the mRNA expression of three transcription factors that are characteristic of pluripotent cells (339): *POU5F1* (OCT4), *NANOG*, and *SOX2* (Fig. 26a). While neither MCF7 nor T47D cells displayed increased *POU5F1* expression following OSM treatment, both cell lines increased expression of *SOX2*; however, this was reproducibly significant only for MCF7 cells ($P<0.05$ in 3/3 replicate experiments). *NANOG* was consistently upregulated only in T47D cells.

The ability to form mammospheres (three dimensional colonies derived from single cells in adhesion-free and defined growth factor conditions) is established as an *in vitro* assay for CSC features and tumourigenicity in breast cancer cells (49, 51, 340). To determine the effect of OSM on mammosphere formation, we cultured MCF7 and T47D cells for 2 days with or without OSM before seeding cells at clonal density under mammosphere forming conditions (OSM-pretreated cells received continuous OSM stimulation throughout the assay). After 10 days of culture, OSM-treated cells were clearly enriched for mammosphere forming ability (Fig. 26b,c). In control MCF7 cultures, the average proportion of mammosphere forming cells was 0.57% (range 0.31%–087%) versus 2.93% (range 1.87%–3.61%) in OSM-stimulated cultures. Similar results were obtained using T47D cells, which displayed an average baseline proportion of 0.34% (range 0.32%–0.36%) compared to an OSM-stimulated proportion of 7.27% (range 7.11%–7.39%). OSM-induced fold changes in mammosphere forming capacity ranged from 4.17–6.38 in MCF7 cells and 20.3–23.1 in T47D cells. Data from a representative experiment is shown in Figure 26c. OSM-stimulated mammospheres were also significantly larger in size than those arising under control conditions ($P<0.0001$ for both cell lines; Fig. 26d). Intriguingly, cells that received only OSM pre-treatment before plating in mammosphere assays also had enhanced mammosphere-forming capacity, indicating that persistent OSM stimulation is not required in this process (data not shown).

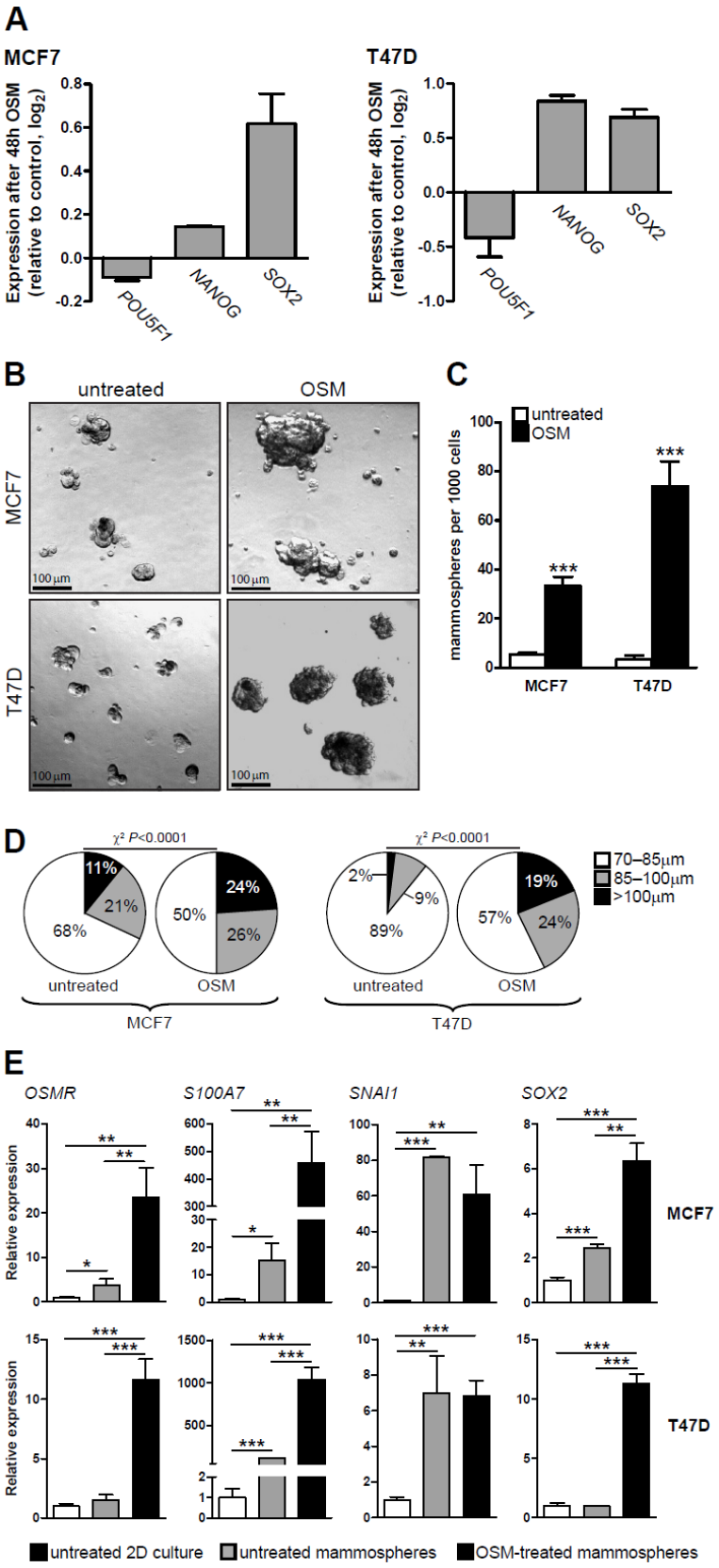


Figure 26. Induction of stem cell features following OSM treatment. (**A**) Q-PCR analysis of pluripotency gene expression in MCF7 and T47D cells following OSM stimulation. Data are expressed relative to untreated cultures. (**B**) Representative images of 10-day mammosphere cultures with or without OSM. (**C**) Quantification of mammosphere formation rates in a representative assay. Bars represent means (+/- S.D.) from four replicate cultures. (**D**) Size distribution of mammospheres formed after 10 days of culture. Data represent the pooled results from 3 independent assays. Statistical significance was established using the χ^2 test. (**E**) Representative Q-PCR analyses of gene expression in control or OSM-stimulated mammospheres relative to pooled samples from 3 independent standard cultures (conventional adherent culture with complete medium). Bars represent means (+/- S.D.) from triplicate assays. In panels C and E, asterisks denote statistical significance determined by Student's t-tests.

We next assessed the expression levels of specific genes in control and OSM-induced mammospheres relative to conventionally cultured cells (i.e., 2-D adherent growth with 5% serum). *OSMR* is a transcriptional target of OSM signalling and was highly expressed in OSM-stimulated mammospheres (Fig. 26e). *S100A7* was highly expressed in these cells, as well as in control mammospheres (albeit at lower levels), suggesting that S100A7 may be inherently related to CSC phenotypes. As expected, *SNAI1* was highly expressed in mammospheres, but showed no consistent changes with respect to OSM treatment. Although both *POU5F1* and *NANOG* were induced to a variable extent in mammospheres, neither showed an association with OSM treatment (data not shown). *SOX2*, however, was expressed at consistently high levels in OSM-treated mammospheres. Given its recent identification as an essential factor for mammosphere formation (341), *SOX2* induction may at least partly explain the mammosphere promoting effect of OSM.

4.5.3—OSM effects are mediated by OSMR

We have previously shown that OSM induces suppression of ER by signalling through OSMR rather than LIFR (334). Knockdown of OSMR expression in MCF7 cells using siRNA confirmed that OSMR is required for OSM-induced expression of *SNAI1*, *SNAI2*, and *SOX2* (Fig. 27). In contrast, while *S100A7* induction was significantly inhibited by OSMR siRNA, OSM nevertheless induced high expression of *S100A7*, suggesting that S100A7 induction may require a relatively low level of OSMR signal strength.

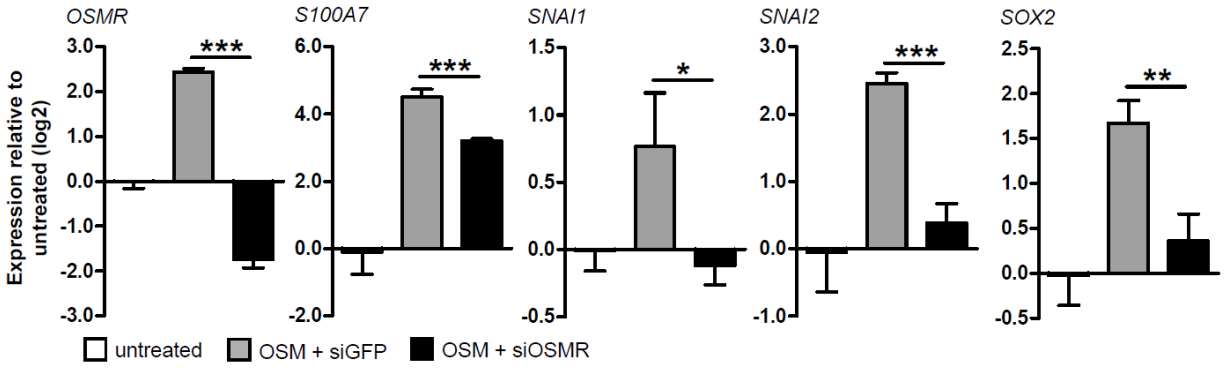


Figure 27. **Blockade of OSM signalling via OSMR knockdown**. MCF7 cells were transfected with control (GFP) or OSMR siRNA for 2 days before stimulation with OSM and harvesting for Q-PCR analysis. Bars represent means (+/- S.D.) from representative assays, expressed relative to untreated control samples (compared using the Student's t-test).

4.5.4—OSM signalling promotes a CD44^{high}CD24$^{-/low}$ cell surface phenotype

The primary immunophenotypic assay for identifying putative mammary CSCs is detection of high CD44 and low or negative CD24 (CD44^{high}CD24$^{-/low}$) cell surface expression by flow cytometry (47, 48). We assessed MCF7 and T47D cells in this manner as an alternative CSC assay to complement our mammosphere data. Both cell lines were largely CD24-positive, while CD44 expression was more variable: roughly 82% of MCF7 cells and 38% of T47D cells were positive for CD44 (Fig. 28a). Because the optimal staining thresholds for identifying tumourigenic CD44^{high}CD24$^{-/low}$ cells are unclear (47), we elected to draw gates on control samples from each cell line to isolate a CD44^{high}CD24$^{-/low}$ population that comprised the same proportion of cells observed to form mammospheres under control conditions (i.e., 0.57% for MCF7 and 0.34% for T47D). These gates were then applied to cells that had been treated for 2 days with OSM. OSM-treated cells in both cell lines displayed increased CD44 expression such that the proportion of gated CD44^{high}CD24$^{-/low}$ cells increased approximately 6-fold (P=0.007) and 22-fold (P=0.001) in MCF7 and T47D cells, respectively (Fig. 28a,b). These values are very consistent with the OSM-induced fold-changes in mammosphere formation noted above (Fig. 26c). Inhibition of PI3K signalling using the inhibitor LY294002 completely abrogated the OSM-induced increase in CD44^{high}CD24$^{-/low}$ cells (Fig. 28b). This was due to inhibition of CD44 expression and enhancement of CD24 levels in both cell lines (Fig. 28c). Treatment with the MEK1/2 inhibitor U0126 caused a similar suppression of the CD44^{high}CD24$^{-/low}$ phenotype by enhancing CD24 expression, without any apparent effect on CD44 levels (Fig. 28b,c).

Flow cytometric assessment of OSMR expression in these assays indicated a fold increase of 2–2.5 in both cell lines following OSM treatment (Fig. 28d), consistent with the inductive effect of OSM on *OSMR* mRNA (Fig. 27). Notably, OSMR expression was correlated with CD44, such that OSM induced a considerable increase in CD44^{high}OSMR^{high} cells (Fig. 28e). The enrichment of CD44^{high}CD24$^{-/low}$ cells following OSM treatment was also more pronounced in cells that exhibited high OSMR levels (data not shown).

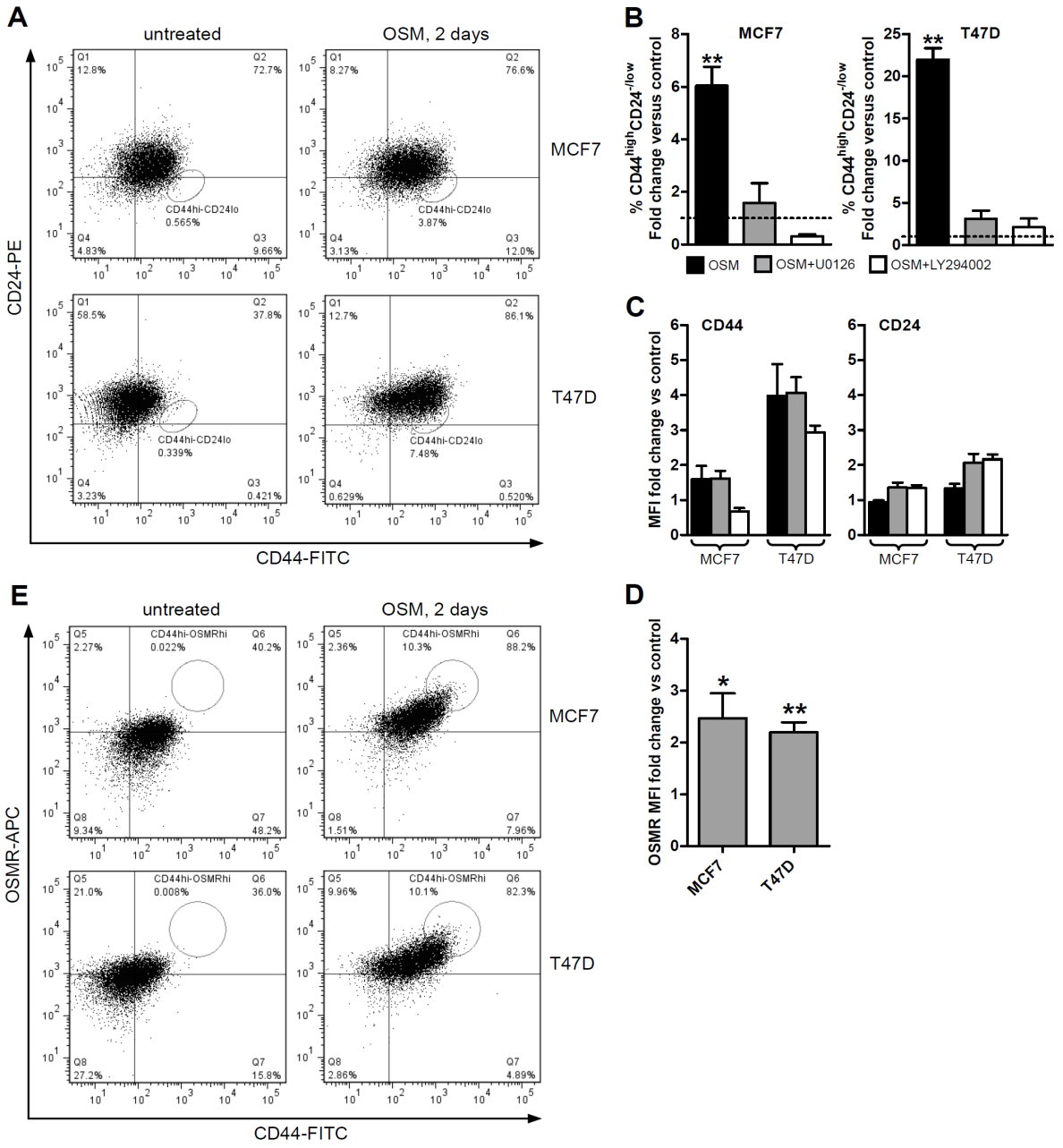


Figure 28. **Flow cytometry analysis of MCF7 and T47D cells**. (**A**) Representative data plots of control and OSM-treated cells stained for CD24 and CD44. Quadrant gates denote thresholds for positive staining based on negative staining controls. Elliptical gates denote $CD44^{high}CD24^{-/low}$ cells. (**B**) Fold changes (relative to control cultures) in the proportion of cells with a $CD44^{high}CD24^{-/low}$ phenotype following 2 days of OSM stimulation, with or without inhibitors to MEK1/2 (U0126) or PI3K (LY294002). (**C**) CD44 and CD24 and (**D**) OSMR MFI fold changes following OSM stimulation. (**E**) As in panel A, representative plots showing CD44 and OSMR expression. Elliptical gates were drawn on plots from OSM-treated cells to denote the strongest 10% in terms of CD44-OSMR coexpression. All bars represent means (+/- S.D.) of 3 independent assays, with significance established by one-sample t-test (versus a hypothetical mean of 1).

4.5.5—The OSM pathway is associated with EMT/CSC features in human tumours

To determine if the OSM pathway in human tumours is associated with features of EMT and CSCs, we used Q-PCR to profile a cohort of 72 human invasive breast carcinomas for expression of *OSM*, *OSMR*, *SNAI1*, *POU5F1*, *SOX2*, and *S100A7*. Data for these genes were median normalized and cases were separated on the basis of high and low expression of *SNAI1*, *POU5F1*, or *SOX2* using the upper quartile as a cutpoint. Although cases with high expression of these genes tended to have higher expression of *OSMR*, this was not statistically significant (data not shown). However, cases high in *SOX2* or *SNAI1* were significantly enriched for expression of *OSM*, and *POU5F1*-high cases had a non-significant trend towards higher *OSM* expression (Fig. 30a). Consistent with the results from our snail siRNA experiment (Fig. 25e), *S100A7* levels were significantly related to *OSM* and *SNAI1* (Fig. 30b); however, no association was seen with *POU5F1* or *SOX2* (data not shown).

We expanded on these observations using a larger publically available dataset. This included microarray gene expression data from fine needle aspirates of 278 breast carcinomas that were subsequently treated with neoadjuvant anthracycline-taxane based chemotherapy (MAQC cohort (335)). Because fine needle aspirates are highly enriched for neoplastic cells, we only assessed the expression of *OSMR* relative to a panel of EMT/CSC-related genes (OSM is thought be derived from leukocytes and could thus give misleading results in this dataset). *OSMR* expression correlated significantly with 9 of 10 EMT genes including strong ($P<0.0001$) positive relationships with *CDH2* and *ZEB2* (also known as SIP1) and a strong inverse relationship with *CDH1* (Table 4). Significant relationships were also observed with each CSC factor we assessed, including the ratio of *CD44* to *CD24*, the pluripotency genes *SOX2*, *POU5F1*, and *NANOG*, members of the *NOTCH* family, and the transmembrane drug transporter *ABCG2*. Although *ALDH1A1* has been associated with breast CSCs, its expression correlated with neither *OSMR* nor other CSC markers in this dataset.

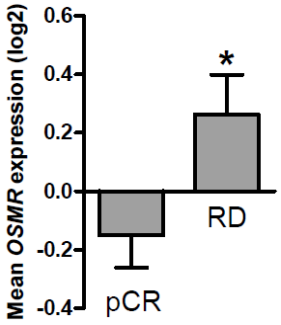


Figure 29. **Association of *OSMR* with chemo-resistance in the MAQC cohort**. Average *OSMR* expression (+/- S.E.M.) in the ER– subset of the MAQC cohort is shown. Cases are divided into those that achieved a pathological complete response (pCR) versus those with residual disease (RD) following neoadjuvant chemotherapy. Significance was calculated using the Mann-Whitney U-test.

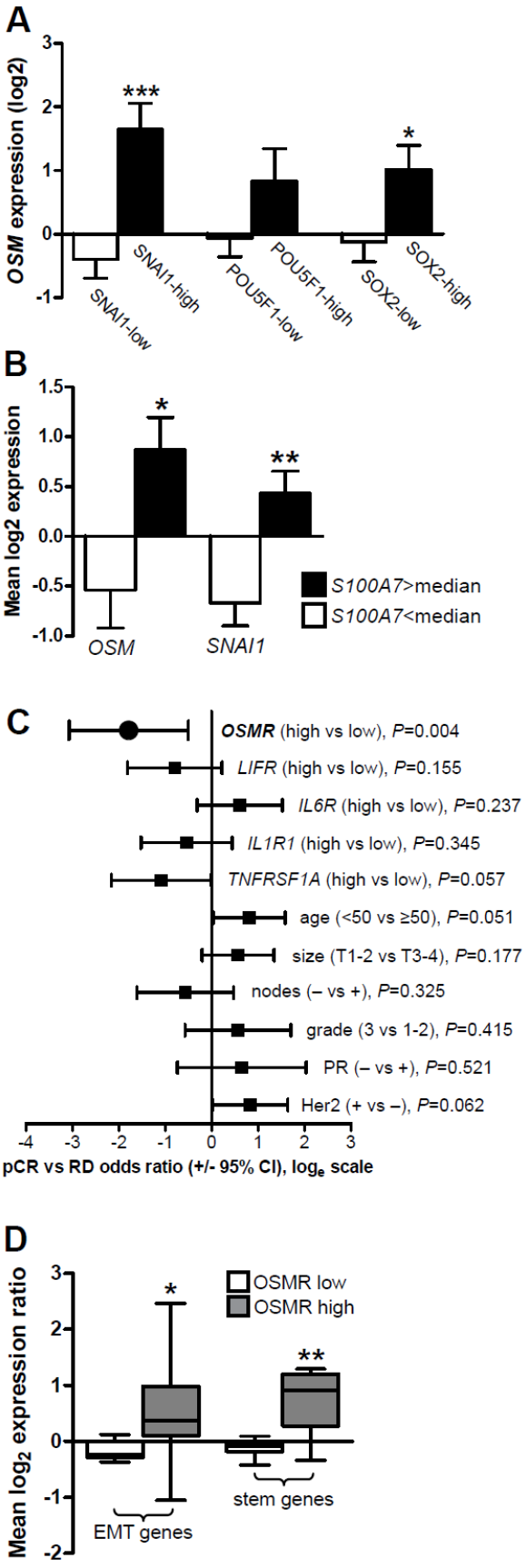


Figure 30. Association of the OSM pathway with EMT/CSC features *in vivo*. (**A**, **B**) Q-PCR assessment of *OSM*, *SNAI1*, *POU5F1*, *SOX2*, and *S100A7* expression in 72 invasive breast carcinomas. Bars represent mean values +/- S.E.M. (**A**) *OSM* expression in cases divided into high and low groups based on expression of *SNAI1*, *POU5F1*, and *SOX2* (cut at the upper quartiles). (**B**) *OSM* and *SNAI1* expression based on *S100A7* levels. (**C**) Logarithmic odds ratios depicting the association of *OSMR*, other cytokine receptors, and clinical parameters with probability of pathological complete response versus residual disease following neoadjuvant chemotherapy in a cohort of 114 ER– breast carcinomas (MAQC cohort). Negative values indicate reduced odds of clinical response. Ratios and significance were determined by Fisher's exact tests. (**D**) Box plots representing the average expression values of genes listed in Tables 4 and 5 within *OSMR* high and low groups from the ER– MAQC cohort. For panels A, B, and D, significance was calculated using the Mann-Whitney U-test.

Table 4. Correlation of *OSMR* with EMT and CSC-associated genes in the MAQC cohort (*n*=278).

Category	Gene	Spearman r	95% CI	*P*-value
EMT factor	*ZEB2*	0.3383	0.2264–0.4413	<0.0001
EMT factor	*CDH2*	0.3292	0.2167–0.4330	<0.0001
EMT factor	*FOXC1*	0.3220	0.2091–0.4265	<0.0001
EMT factor	*CDH1*	-0.3213	-0.4258– -0.2083	<0.0001
EMT factor	*ZEB1*	0.2697	0.1536–0.3784	<0.0001
EMT factor	*SNAI2*	0.2487	0.1316–0.3590	<0.0001
EMT factor	*FOXC2*	0.1821	0.06240–0.2967	0.0023
EMT factor	*VIM*	0.1781	0.05821–0.2929	0.0029
EMT factor	*SNAI1*	0.1283	0.0073–0.2456	0.0325
EMT factor	*TWIST1*	-0.0165	-0.1373–0.1048	0.7842
CSC factor	*ABCG2*	0.4564	0.3549–0.5473	<0.0001
CSC factor	*EGFR*	0.4156	0.3101–0.5110	<0.0001
CSC factor	*NOTCH4*	0.3950	0.2877–0.4926	<0.0001
CSC factor	*SOX2*	0.3875	0.2795–0.4858	<0.0001
CSC factor	*CD133*	0.2890	0.1740–0.3962	<0.0001
CSC factor	*CD44/CD24**	0.2625	0.1460–0.3718	<0.0001
CSC factor	*NOTCH1*	0.2279	0.1098–0.3396	0.0001
CSC factor	*NANOG*	0.2168	0.0983–0.3293	0.0003
CSC factor	*POU5F1*	0.1713	0.0513–0.2865	0.0042

**CD44/CD24* denotes the ratio of *CD44* to *CD24* expression.

Because CSCs are thought to contribute to therapeutic resistance in breast cancer, we examined the relationship of *OSMR* expression with rates of pathological complete response (pCR) in the ER– subset of the MAQC cohort. The term pCR refers to the complete absence of neoplastic cells during pathological assessment of post-chemotherapy surgical resections. We focused on ER– cases because the vast majority of pCRs following neoadjuvant chemotherapy are achieved by ER– patients (72, 342-344) and *OSMR* expression is associated with ER– status. Indeed, ER– status was a strong predictor of pCR in this dataset (Fisher's exact test, $P<0.0001$) and high *OSMR* expression (upper quartile) was associated with ER– tumours ($P<0.05$). *OSMR* expression was significantly increased in ER– patients that did not achieve a pCR ($P=0.03$; Fig. 29). To determine the optimal *OSMR* cutpoint for predicting pCR we performed receiver-operator characteristic (ROC) analysis, which led us to define *OSMR*-high status as the upper 20% of cases. *OSMR*-high patients had a pCR rate of 3/23 (13%), while 43 of 91 (47%) *OSMR*-low patients achieved pCR (OR=0.167, 95% CI 0.046–0.603; $P=0.004$). To determine if other cytokine receptors were related to chemotherapy responsiveness we performed a similar analysis (using the upper 20% to define 'high' status) on

LIFR, *IL6R*, *IL1R1*, and *TNFRSF1A*. None of these genes were significantly associated with pCR rates, although trends toward worse outcome were seen with *TNFRSF1A* and, to a lesser extent, *LIFR* (Fig. 30c). No standard clinical parameters were significantly associated with pCR in this dataset. Many of the genes listed in Table 4 that correlated with *OSMR* in the whole dataset also correlated significantly with *OSMR* in the ER– subset (Fig. 30d; Table 5). Notably, the only three pCR cases in the *OSMR*-high group were Her2-amplified. When Her2+ and PR+ cases were excluded to focus on the clinically important triple negative subset, pCR occurred in 0 of 17 *OSMR*-high cases and 25 (46%) of 54 *OSMR*-low cases (OR=0.033, 95% CI 0.002–0.578; *P*=0.0003). Again, EMT and CSC genes were enriched in triple negative, *OSMR*-high cases (data not shown). Genes that correlated strongly with *OSMR* in triple negative cases included *ZEB2* (r_s=0.5876, *P*<0.0001), the *CD44*/*CD24* ratio (r_s=0.4431, *P*=0.0001), *SOX2* (r_s=0.4380, *P*=0.0001), *ABCG2* (r_s=0.4370, *P*=0.0001), *CDH1* (r_s=-0.4317, *P*=0.0002), and *NOTCH4* (r_s=0.4142, *P*=0.0003).

Table 5. Correlation of *OSMR* with EMT/CSC-related genes in the ER– subset of the MAQC cohort (*n*=114).

Category	Gene	Spearman r	95% CI	*P*-value
EMT factor	*ZEB2*	0.4664	0.3039–0.6025	<0.0001
EMT factor	*CDH2*	0.3657	0.1895–0.5190	<0.0001
EMT factor	*FOXC1*	0.2221	0.0343–0.3948	0.0175
EMT factor	*CDH1*	-0.3886	-0.5383– -0.2152	<0.0001
EMT factor	*ZEB1*	0.3037	0.1215–0.4662	0.0010
EMT factor	*SNAI2*	0.1617	-0.0284–0.3406	0.0856
EMT factor	*FOXC2*	0.2701	0.0852–0.4370	0.0037
EMT factor	*VIM*	0.0715	-0.1194–0.2573	0.4497
EMT factor	*SNAI1*	0.1225	-0.0684–0.3047	0.1942
EMT factor	*TWIST1*	0.0467	-0.1438–0.2339	0.6215
CSC factor	*ABCG2*	0.4565	0.2925–0.5944	<0.0001
CSC factor	*EGFR*	0.3804	0.2059–0.5314	<0.0001
CSC factor	*NOTCH4*	0.4046	0.2332–0.5516	<0.0001
CSC factor	*SOX2*	0.3554	0.1781–0.5103	0.0001
CSC factor	*CD133*	0.1885	-0.0008–0.3647	0.0446
CSC factor	*CD44*/*CD24**	0.3790	0.2043–0.5302	<0.0001
CSC factor	*NOTCH1*	0.1401	-0.0505–0.3209	0.1370
CSC factor	*NANOG*	0.3098	0.1280–0.4714	0.0008
CSC factor	*POU5F1*	0.2961	0.1132–0.4596	0.0014

**CD44*/*CD24* denotes the ratio of *CD44* to *CD24* expression.

4.5.6—The association of OSMR with poor long-term prognosis depends on coexpression of genes related to EMT and CSCs

We have previously shown that high expression of *OSMR* is associated with poor 5-year disease-free survival (DFS) in a separate publically available dataset (Prat cohort (23); Chapter 3). We revisited this cohort to determine if coexpression of EMT/CSC-related genes contributed to this finding. Cases were sorted into four groups on the basis of high or low *OSMR* (cut at the upper quartile) and coexpression of 4 or more of a panel of 6 EMT/CSC-relevant genes (using their respective medians to define positive expression). The smaller number of assessed genes in this cohort reflects the lower number of probe sets that passed quality filtering steps. The genes used were *SNAI1*, *CDH2*, *EGFR*, *NOTCH1*, *CD133*, and *POU5F1*. Patients with low *OSMR* and low EMT/CSC gene expression had a favourable 5-year DFS rate of 70% (n=96; Fig. 31a). The two patient categories with high levels of either *OSMR* or EMT/CSC gene expression had indistinguishable outcomes (P=0.99) and were thus combined to form a single intermediate group with a 5-year DFS rate of 55% (n=48). Cases with high levels of both *OSMR* and EMT/CSC gene expression had a dismal 5-year DFS rate of only 17% (n=27; P<0.0001, and log-rank test for trend P_T<0.0001). Though not included in the 6-gene EMT/CSC module (see Methods), *CD44* was strongly expressed in poor prognosis patients, while *CDH1* expression showed the opposite trend (Fig. 31b). As expected, coexpression of the EMT/CSC panel was associated with *OSMR*-high status (Fig. 31c).

Because we found that PI3K was essential for OSM to promote a $CD44^{high}CD24^{-/low}$ phenotype *in vitro*, we assessed the relationship between PI3K signalling and OSMR in the Prat cohort. As an indicator of PI3K activity, we utilized the breast cancer PI3K metagene identified by Creighton *et al* (116). When cases were categorized based on expression of *OSMR* and the PI3K metagene, those with high expression of each (n=26) had higher average expression of the 6-gene EMT/CSC panel than any other group (Fig. 31d). These cases also had much worse prognosis than any other group (Fig. 31e), indicating that high levels of EMT/CSC gene expression and poor prognosis occurs in the context of high *OSMR* expression and an active PI3K pathway. Intriguingly, *S100A7*, a PI3K-dependent target gene of OSM and a mediator of OSM-induced migration (Chapter 2 (309)), was similarly associated with coexpression of *OSMR* and the PI3K metagene (Fig. 31f). These data thus support the premise that an important action of OSM signalling is to induce or enhance a mesenchymal/CSC phenotype in breast cancer cells through a mechanism that may be dependent on PI3K signalling.

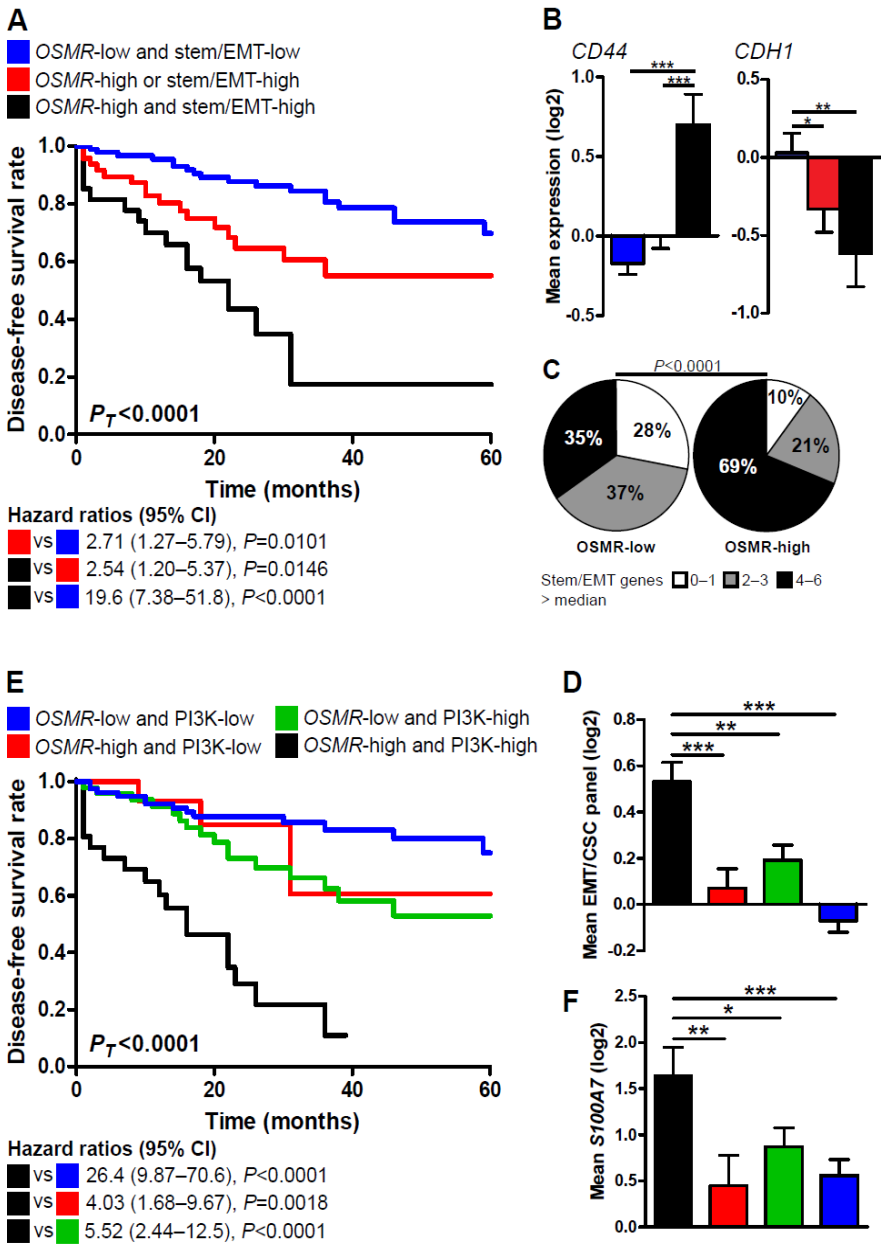


Figure 31. Relationship between *OSMR* expression, EMT/CSC-associated genes, and 5-year prognosis. All panels show data derived from the Prat cohort. (**A**) Disease-free survival of cases categorized by *OSMR* and coexpression of 4 or more of the following genes: *EGFR*, *NOTCH1*, *CD133*, *SNAI1*, *CDH2*, and *POU5F1*. (**B**) Expression of *CD44* and *CDH1* in the three groups from panel A. (**C**) Levels of coexpression of the 6 EMT/CSC genes in *OSMR* high and low groups (significance calculated by χ^2 test). (**D**–**F**) Assessment of gene expression and survival in cases categorized by *OSMR* levels and expression of a PI3K signalling metagene. The legend in panel E corresponds to panels D through F. (**D**) Average expression of the 6-gene EMT/CSC panel and (**E**) DFS in the indicated groups. Corresponding *S100A7* expression levels are shown in panel **F**. Survival curve statistics were calculated using the log-rank test. All gene expression bar charts represent mean levels +/- S.E.M., with significance established using the Mann-Whitney U-test.

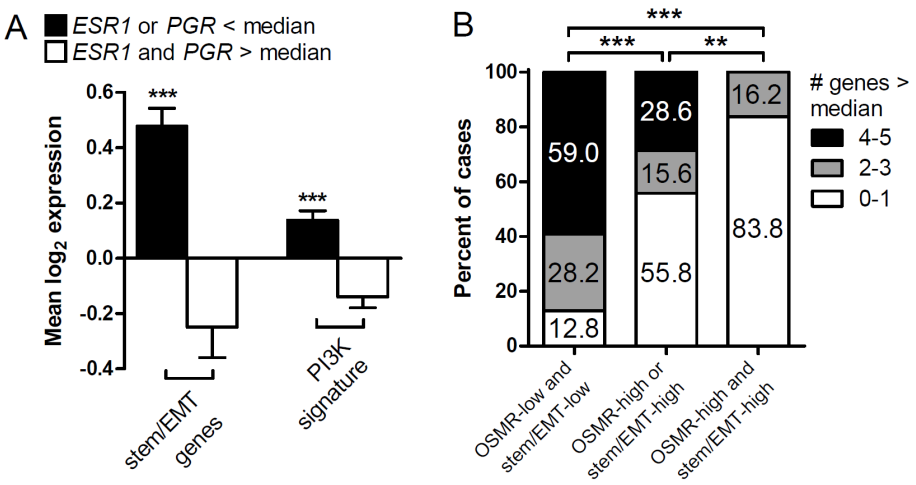


Figure 32. **Relationship between hormone receptor expression, EMT/CSC genes, and *OSMR***. Data is derived from the Prat cohort. (**A**) Mean (+/- S.E.M.) expression of the 6-genes that comprise the EMT/CSC module in *OSMR*-high patients with (*n*=9) or without (*n*=49) retention of *ESR1* and *PGR* coexpression. Significance was calculated using the Mann-Whitney U-test. (**B**) Expression of the 5-gene ER module in tumours categorized by *OSMR* and EMT/CSC module expression, analyzed using the χ^2 test.

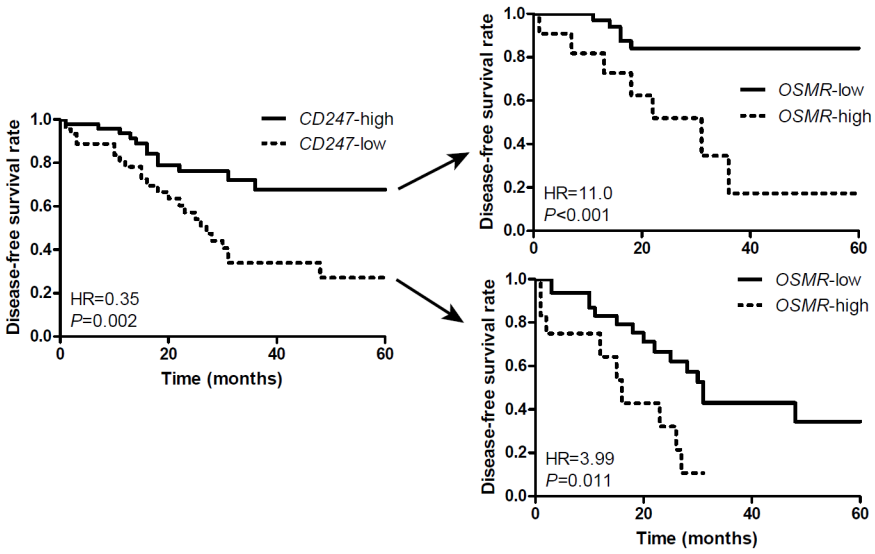


Figure 33. **Prognostic impact of *OSMR* and T cell infiltration in ER– tumours**. DFS of ER– Prat cohort cases categorized by high (>median; *n*=49) and low (*n*=45) expression levels of the T cell-specific gene *CD247* (CD3ζ). These groups are further divided based on *OSMR* expression. The upper quartile was used to define *OSMR*-high status. Survival curve statistics were calculated using the log-rank test. High/low cutpoints reflect the data distribution of ER– tumours (there were 11 *OSMR*-high cases in the *CD247*-high group and 12 in the *CD247*-low group).

We previously demonstrated that OSM signalling is associated with loss of *ESR1* and *PGR* expression, and that rare *OSMR*-high cases with high (> median) *ESR1* and *PGR* expression have a good prognosis (Fig. 21). Re-analysis of *OSMR*-high cases in the Prat cohort revealed that the minority of cases with *ESR1* and *PGR* coexpression had low expression of both the 6-gene EMT/CSC module and the PI3K signature (Fig. 32a). Similarly, assessment of the five ER-module genes described in Chapter 3 (*ESR1*, *PGR*, *CCND1*, *GATA3*, and *TFF1*) in the three patient categories shown in Figure 31a revealed low levels of coexpression in the poor prognosis group defined by high *OSMR* and EMT/CSC module expression (Fig. 32b). Collectively, these data suggest that the majority of *OSMR*-high tumours have both high expression of EMT/CSC-associated genes and low expression of ER-regulated genes, and that tumours with these features are primarily responsible for the relationship between *OSMR* and poor prognosis.

Tumour infiltrating lymphocytes (TIL) are associated with improved prognosis and sensitivity to chemotherapy in ER– breast cancer (130-132, 146). Given that OSM is likely derived from several possible leukocyte subsets in breast tumours, this suggests that expression of OSMR by tumour cells could enhance their resistance to immune attack. Using *CD247* (CD3ζ) as a marker of T cells, we confirmed that T cell infiltration in the ER– subset (n=94) of the Prat cohort was associated with good prognosis (Fig. 31g). High expression of *OSMR*, however, was a poor prognostic factor regardless of T cell infiltration (P<0.001 and P=0.011 in T cell high and low groups, respectively). Furthermore, high *CD247* expression did not significantly improve the outcome of *OSMR*-high cases, but was strongly prognostic for *OSMR*-low tumours (P=0.003, data not shown). OSM signalling may thus be a mechanism of immune evasion in breast cancer.

4.6—Discussion

The expression of a mesenchymal or stem cell-like phenotype by malignant cells is increasingly thought to promote treatment resistance and metastasis. We have shown for the first time that OSM can induce features consistent with an EMT/CSC phenotype in models of luminal breast cancer. Furthermore, in human breast cancer cohorts, the OSM pathway is consistently related to EMT/CSC features, which may explain the relationship between OSMR expression and poor prognosis.

Our assessment of EMT features induced by OSM revealed that OSM can trigger many, but not all of the changes that are classically associated with EMT (38). These include (in addition to the pro-migratory effect of OSM described by several groups) loss of epithelial morphology and suppression of epithelial markers such as E-cadherin and hormone receptors (334), deregulation of

β-catenin, and gain of several transcriptional regulators of EMT, most notably snail and slug. However, the variation between MCF7 and T47D cells and the unexpected suppressive effect of OSM on vimentin suggests that OSM treatment does not cause a complete EMT, but rather regulates a subset of EMT features in a context-dependent manner. Indeed, our observations are perhaps better interpreted as demonstrating carcinoma dedifferentiation, rather than complete transdifferentiation to a mesenchymal state (40, 42). Several lines of evidence suggest that EMT does not occur to completion during cancer progression, including the phenotypic similarity of metastases to primary tumours (37, 345), the expression of epithelial markers by disseminated bone marrow-resident tumour cells (52), the conventional use of cytokeratins and EpCAM to identify putative circulating tumour cells (35), and the possible requirement for re-expression of epithelial features in proliferating metastases (the so-called mesenchymal-to-epithelial transition (37, 45)).

The recent work of Dubois-Marshall *et al* (41) provides an excellent demonstration of the complex expression of EMT markers *in vivo*: in two cohorts totaling over 200 invasive breast carcinomas (mostly high grade), they used quantitative immmunofluorescence to assess several EMT markers including E- and N-cadherin, β-catenin, fibronectin, vimentin, snail, and slug. In contrast with conventional models of EMT, they observed no clear correlation between loss of E-cadherin and gain of N-cadherin, nor were significant correlations observed between N-cadherin, vimentin, and fibronectin expression. However, snail and slug expression were correlated in both datasets, without any apparent relationship to the other markers assessed, including E-cadherin. This bears a striking resemblance to our data, which revealed consistent upregulation of snail and slug following OSM treatment, but few consistent trends for other EMT markers.

Intriguingly, while OSM triggered a reduction of E-cadherin membrane localization in both MCF7 and T47D cells, this was attributable to reduced mRNA levels in the former and protein internalization in the latter (although this may have led to eventual E-cadherin degradation after extended OSM treatment (346)). Expression of snail and/or slug is conventionally thought to induce E-cadherin suppression, based on both experimental data and clinical correlations (23, 336, 338). However, the maintenance of E-cadherin mRNA and protein levels in T47D cells following OSM stimulation, despite the robust increase in snail and slug expression, demonstrates that E-cadherin repression is not a universal effect of these transcription factors. This conclusion was also drawn by Côme *et al* (347), who observed frequent coexpression of E-cadherin, snail, and slug in a cohort of human breast tumours. Notably, E-cadherin is essential for maintenance of a pluripotent state (348). It may therefore be advantageous for cancer cells to suppress E-cadherin only to the point of

enhancing migration and invasion, such that its expression can be readily restored to support a stem-like phenotype.

The induction of EMT has been mechanistically linked to the acquisition of stem cell-like properties, including an enhanced ability to promote mammary gland outgrowth and tumour formation in non-transformed and transformed cells, respectively. Two of the most common approaches for identifying putative mammary CSCs, both of which are validated methods for identifying cells with enhanced tumourigenic capacity, are the propagation of single cells in defined culture to form mammospheres (49, 51, 340) and detection of a $CD44^{high}CD24^{-/low}$ cell surface phenotype (47, 48). In our hands, OSM promoted both MCF7 and T47D mammosphere formation as well as increased levels of $CD44^{high}CD24^{-/low}$ cells. Within each cell line, the fold changes in mammosphere formation and $CD44^{high}CD24^{-/low}$ phenotype induction following OSM treatment were nearly identical, providing strong evidence that OSM augments features consistent with a mammary CSC phenotype.

Intriguingly, the only pluripotency transcription factor that was reproducibly induced by OSM was SOX2, an essential factor for the generation of pluripotent stem cells from fibroblasts (349). This was observed both in conventional culture conditions (primarily for MCF7 cells) and in mammosphere assays (for both cell lines). Although OCT4 and NANOG are both associated with pluripotency, it was recently shown that SOX2 was the only one of these three factors to be expressed at high levels in early breast tumours and was required for efficient mammosphere and xenograft formation by MCF7 cells (341). Furthermore, SOX2 appears to be preferentially expressed in basal-like breast cancers (350), which are known to exhibit poor differentiation and a poor prognosis. In addition to SOX2, OSM promoted high levels of S100A7 expression in both MCF7 and T47D mammospheres. S100A7 is known to promote anoikis resistance and tumourigenesis in ER– breast cancer cells and may be required for OSM-induced migration (272, 278, 309). Given its relatively high expression in both OSM-induced and untreated mammospheres, S100A7 may be inherently related to CSC biology. Finally, OSMR expression was heightened in OSM-induced mammospheres and was strongly coexpressed with CD44 (determined by flow cytometry), suggesting that high OSMR levels may be associated with cytokine-induced stemness.

The precise mechanism by which OSMR signalling promotes an EMT/CSC phenotype is unclear, but it likely relies on all three of the major downstream pathways, namely, STAT3, MAPK, and PI3K. In this study we identified PI3K and, to a slightly lesser extent, MAPK signalling as essential for adoption of a $CD44^{high}CD24^{-/low}$ phenotype. Expression of a PI3K gene expression

signature in the context of OSMR expression also seemed to be clinically relevant, consistent with the role of PI3K in CSC biology reported previously (351-354). In cooperation with PI3K and mTOR, STAT3 was shown to be required for the expression of stem cell features and *in vivo* tumourigenicity of MCF7 cells (354). In mice, Her2-driven mammary tumours deficient in STAT3 are attenuated in their metastatic capacity and, intriguingly, display markedly reduced levels of OSMR (355). STAT3 is known to induce OSMR expression in the mammary gland and is important for the pro-migratory effect of OSM on breast cancer cells (235, 356). Intriguingly, both OSMR and gp130 can directly interact with EGFR family receptors to engage in cooperative signalling (184, 234). Because EGF is a key component of mammosphere culture media, this could be an important aspect of the mammosphere-promoting effect of OSM. Finally, our attempt to block β-catenin expression using siRNA caused high levels of cell death upon stimulation with OSM, implicating it as a potentially important mediator of cell integrity during cytokine stimulation.

Our *in vitro* observations were nicely borne out in cohorts of human breast cancer. These collectively demonstrated that OSM and/or OSMR are related to genes associated with EMT and stemness *in vivo*. Although the Prat dataset lacked informative data for several genes of interest (notably SOX2), it nevertheless demonstrated that the previously observed association between OSMR expression and poor prognosis is likely related to coexpression of EMT/CSC factors. It could be argued that OSMR correlates with prognosis due to its enrichment in ER– tumours. It was indeed the case that high OSMR and EMT/CSC factor coexpression was associated with reduced ER pathway expression (Fig. 32), but we have shown previously in this cohort that OSMR has prognostic independence from clinical ER status and other histopathological factors based on multivariate analysis (334). Furthermore, the association of high OSMR expression with poor response to chemotherapy in ER– tumours within the MAQC cohort illustrates that OSMR can be related to poor clinical outcome even in cohorts enriched for aggressive tumours.

We have recently reported that a high level of tumour infiltrating lymphocytes is strongly predictive of complete clinical response to neoadjuvant anthracycline-based chemotherapy in ER– breast cancer (146), and others have shown that TIL are related to good prognosis in this disease subtype (130-132). However, Figure 33 demonstrates that ER– tumours with high OSMR expression may be inherently resistant to anti-tumour immunity. Although little is known regarding the precise immunological relevance of cancer EMT and CSCs, observations that $CD8^+$ T cells can induce the evolution of murine mammary tumours with mesenchymal/CSC phenotypes (57) and that snail expression in melanoma inhibits anti-tumour immunity (357) suggest that adoption of an

EMT/CSC phenotype is a pertinent mechanism of immune escape and subversion. The ability of breast tumours to exploit OSM is therefore a possible hazard that should be considered when designing and deploying cancer immunotherapies.

A conceptually attractive aspect of OSM signalling is the rapidity with which it exerts its effects. Many studies that identify factors with a role in EMT and/or CSC biology depend on constitutive expression strategies, raising questions regarding the true physiological relevance of the findings. This has been the case for many intracellular factors such as snail, twist, and SOX2 (43, 341), as well as exogenous factors like IL-6 (186). An example of where this approach could lead to problems of interpretation is the forcible expression of snail, which under normal circumstances binds to and inactivates its own promoter (336). In some studies involving cytokines that are implicated in EMT/CSC induction, such as TGFβ and TNF, cells were exposed to these factors for lengthy periods of time before EMT/CSC phenotypes were assessed, ranging from 12 to 60 days (43, 44, 60). In contrast, OSM-stimulated breast cancer cells exhibit rapid changes that occur within 24 hours such as loss of ER and gain of S100A7 (309, 334), and manifest a readily detectable EMT/CSC-like phenotype within 48 hours. Effects exerted by IL-6, an OSM family member, may occur with similar kinetics (61, 184). The ability to exert changes on a relatively short time scale may be important in an *in vivo* setting—given that leukocytes (a major source of intratumoural cytokines) are dynamic components of the microenvironment, it is difficult to know how long a single malignant cell within a tumour would be exposed to effective concentrations of immune-derived cytokines such as OSM or TNF. Thus, it may be that relatively fast-acting cytokines like OSM are more likely to exert physiologically meaningful effects during tumour evolution.

To firmly establish OSM as a relevant tumour-promoting factor, two major tasks are required: one, to study the effects of OSM on tumourigenesis in preclinical animal models and two, to firmly establish the relationship between OSM signalling and prognosis in well-designed clinical cohorts. The latter will require the validation of reliable detection methods for OSMR. To date, immunohistochemical OSMR detection has rarely been performed, and has relied on weakly characterized polyclonal antibodies. If the apparent importance of OSM signalling is verified in these settings, OSM and OSMR could represent attractive therapeutic targets for breast cancer.

CHAPTER 5—Concluding remarks

5.1—Chapter summaries and discussion

Breast cancer remains a significant clinical challenge largely because of its broad phenotypic diversity. The respective successes of endocrine and trastuzumab therapy for treating ER+ and Her2+ breast cancers are good examples of the benefits of molecularly targeted approaches. Nevertheless, many eligible patients do not fully benefit from these treatments, such as the roughly 30% of ER+ patients whose tumours eventually develop resistance to endocrine therapy. In this example, resistance can develop with either retention or loss of ER expression, demonstrating that multiple evolutionary pathways can occur in what began as clinically similar tumours. Even more challenging are the so-called triple negative tumours, which can be systemically treated only with conventional cytotoxic chemotherapies. Ultimately, regardless of the tumour phenotype, metastatic breast cancer is almost universally lethal, making improved prevention and management of metastatic disease a crucial objective of breast cancer scientists and clinicians. In order to effectively address these challenges we must improve our understanding of the mechanisms by which breast tumours evolve. In other words, we must answer key questions such as: why do breast cancers exhibit such diversity, how do specific disease phenotypes arise, and how do established breast tumours alter their phenotypes over time?

Breast cancer etiology and evolution is a widely debated subject that has yet to be resolved. Those who support a stem cell-of-origin model argue that the observed diversity of breast cancer is a product of transformative events within distinct stem/progenitor cell populations of the mammary gland (27). This is an attractive concept, as it suggests that elucidation of the normal stem cell hierarchy of the mammary gland could reveal the fundamental biological precursors of distinct tumour phenotypes, thereby providing deeper insights into their biology. Unfortunately, serious exploration of this idea is currently limited by our incomplete knowledge of the mammary stem cell hierarchy. Furthermore, measurable intratumoural diversity is a common phenomenon, whereby a single lesion can contain multiple distinct phenotypes, both in terms of biomarker expression and gene expression profile (98). It seems rather unlikely that such tumours are composed of distinct lineages of cells that arose from mutagenic events in different stem cells. Thus, although breast cancers may indeed arise from mutated stem cells, it is likely that their ultimate phenotypes are dictated not only by their cell of origin but also by their specific combinations of mutations,

epigenetic modifications, and environmental pressures. Indeed, it is now increasingly clear that factors within the tumour microenvironment have considerable influence on breast cancer biology. The diversity of such factors is enormous, including inorganic pressures such as hypoxia and biological ones such as anti-tumour immune responses. As described in Chapter 1, very few reproducible differences are detectable between synchronous *in situ* and invasive breast cancers, whereas changes in the tumour stroma that accompany invasion clearly occur. Even at this early stage, genetically normal cells in the vicinity of the tumour may thus be vital participants in the progression of disease.

A popular approach to modern cancer research is to conduct translational or 'bench-to-bedside' studies, whereby observations derived from basic science are tested in the context of human patients or tissue samples. This thesis presents data derived from the opposite approach, that is, the exploration of puzzling clinical observations via basic science. Specifically, we have directly or indirectly addressed the following questions: (a) why does S100A7, a normal feature of epidermal differentiation, become expressed in malignant breast tissue; (b) why do many breast tumours fail to express ER, and how can ER+ tumours progress to an ER– state; (c) what are the trigger mechanisms that push breast cancer cells into a mesenchymal or stem cell-like state; and (d) why does inflammation tend to associate with all of these features?

5.1.1—Regulation of S100A7 by OSM

In Chapter 2 we demonstrated that OSM is a potent inducer of S100A7 expression in MCF7 and T47D cells, which display no evidence of S100A7 expression under control conditions. This was also the case in MDA-MB-468 cells, which feature endogenous S100A7 production. The mechanistic underpinnings of this effect are useful for understanding why S100A7 is not normally observed in the mammary gland: we found that OSM-mediated S100A7 expression was dependent on the combined effects of STAT3, MAPK, and PI3K signalling, in that abrogation of any one of these mechanisms inhibited S100A7 production (Fig. 7). Furthermore, EGFR signalling also appeared to be involved in S100A7 production, since OSM stimulated the production of EGF and pharmacological blockade of EGFR inhibited S100A7 expression (Fig. 8). Intriguingly, we also found a requirement for snail in S100A7 expression, implicating S100A7 as a potential marker of an EMT-like process (Fig. 25). Although other cytokines such as IL-6, TNF, and IL-1 could induce S100A7 to some extent, the effects of these cytokines were significantly weaker than that of OSM. However, combination of OSM with either TNF or IL-1 caused a synergistic induction of S100A7,

suggesting that activation of canonical NF-κB signalling along with OSM-induced pathways can drive maximal S100A7 expression (Fig. 3). Indeed, S100A7 induction from OSM alone is likely to be at least partially dependent on NF-κB activity (Fig. 17). Our findings collectively suggest that the expression of S100A7 in mammary tissue is tightly regulated via strict signal transduction requirements. Not only must specific stimuli be in place to simultaneously engage the STAT3, MAPK, and PI3K pathways, the additional expression of other regulatory factors such as snail are also required. These conditions are unlikely to be coincident in normal tissue. Furthermore, the response of non-transformed mammary cells to OSM is fundamentally different from that of their malignant counterparts, in that OSM triggers apoptosis in the normal condition (potentially due to differences in c-myc regulation (240, 241)). Therefore, through deregulation of growth factor pathways and the induction of an inflammatory environment, breast tumours foster conditions conducive to S100A7 expression that are not present in the normal mammary gland.

The induction of S100A7 in breast cancer cells following exposure to OSM and other cytokines such as IL-6, IL-1, TNF, and EGF bears a striking resemblance to the data derived from studies of skin inflammation. Indeed, S100A7 is now viewed as a hallmark of inflammatory cytokine signalling in the field of cutaneous immunology. Coupled with previous observations that S100A7 is associated with intratumoural leukocytes (274, 275), it now appears that S100A7 is also an inflammation response gene in breast cancer. Intriguingly, S100A7 expression was recently shown to promote expression of inflammatory cytokines such as IL-1 and IL-8 in MDA-MB-231 cells, and to promote mammary tumourigenesis and metastasis in a syngeneic mouse model through a seemingly macrophage-dependent mechanism (358). Inflammatory signals may thus both induce S100A7 and be regulated by S100A7 in turn. It is unclear how OSM signalling relates to other S100A7-regulatory factors such as metabolic stress (confluence and growth factor deprivation (265)), ERβ (264), c-myc, and BRCA1 (263). Nevertheless, with the exception of general cell stress, links exist between OSM and each of these factors. For example, OSM is known to influence c-myc expression (241), and c-myc has been shown to suppress S100A7 expression in cooperation with BRCA1 (263). In turn, BRCA1 is frequently mutated or suppressed in basal-like breast tumours (22), a subtype enriched for OSM and OSMR expression (Fig. 20), raising the possibility that OSM could exert effects on S100A7 via regulation of c-myc and/or BRCA1 expression. In the case of ERβ, prior work using MCF7 cells demonstrated that specific activation of ERβ but not ERα could augment S100A7 expression (264). In our hands, preliminary data indicate that OSM stimulation of MCF7 or T47D

cells in the absence of ER ligands has no effect on S100A7 expression (data not shown), suggesting that ERβ signalling may indeed be required in this process.

Our data indicate that S100A7 is a functionally important component of OSM signalling in breast cancer, since siRNA-mediated blockade of S100A7 expression prevented OSM-induced migration (Fig. 9). We have also found that constitutive expression of S100A7 augments migration in MDA-MB-231 cells (273). Given the consistent tumour promoting effects of S100A7 in the context of the ER– cell lines MDA-MB-231 and MDA-MB-468 (266, 268, 272), as well as in MCF7 cells downstream of OSM stimulation, it is puzzling that constitutive expression of S100A7 in MCF7 and T47D cells was recently shown to inhibit cell proliferation, migration, and *in vivo* tumourigenesis (359). The authors of this study concluded that S100A7 may possess different activities depending on ER expression status. This seems sensible when one recalls the strong relationship between S100A7 and ER– status *in vivo* and, in the case of our OSM studies, that OSM induces simultaneous S100A7 induction and ER suppression. However, if one assumes that cytokine stimulation is the normal context in which S100A7 is produced, another explanation for their data is that S100A7 overexpression in an ER+ cell in the absence of inflammatory signals is non-physiological. Without the complex signal transduction induced by a cytokine such as OSM, S100A7 may have very different effects. This concept is compatible with the oncogenic effects of S100A7 in MDA-MB-231 and MDA-MB-468 cells, since both of these lines constitutively express IL-6 (360). This concept may also explain why S100A7 appears to be associated with poor prognosis specifically in tumours that express high levels of OSMR (Fig. 11).

5.1.2—Regulation of ER by OSM

The ability of OSM to suppress ER expression and estrogen responsiveness is arguably the most significant observation described in this manuscript (Chapter 3). Although we are not technically the first group to observe this (i.e. Grant *et al* (234) saw this in 2002 but made only a limited observation that was not pursued further), we are the first to characterize the phenomenon in a reasonably robust way. The importance of ER has been described several times thus far in this thesis, so to discuss it yet again would be tiresome. In brief, ER is the essential target of endocrine therapy and its expression is intimately linked to the various known molecular breast cancer subtypes.

Many cytokines are expressed at high levels in ER– tumours (124, 361), consistent with the known relationship between ER-negativity and high leukocyte content (125). Relatively little has been done, however, to directly ascertain whether cytokines are functionally related to ER

suppression in breast cancer. In separate studies, IL-6, IL-1 and TNF have been shown to suppress ER expression (109, 119, 120, 306); in particular, the autocrine IL-6 loop maintained by MDA-MB-231 cells appears to be critical for their constitutive ER suppression. However, other studies indicate that these cytokines may augment ER transcriptional activity, making their true effects unclear (121-123). In our experiments we did not carefully examine the effects of these cytokines on ER expression. However, direct comparison between IL-6 and OSM suggested that OSM was considerably more effective as an ER suppressor (Fig. 13), and OSM suppression of ER reproducibly inhibited the ability of MCF7 and T47D cells to respond to estrogen (Fig. 18, 19). Preliminary data indicates that OSM causes a synergistic suppression of ER when combined with TNF, whereas the combination of IL-6 with OSM has no additional effect over OSM alone (data not shown). Importantly, our analyses of clinical data suggested that OSM and/or OSMR expression are more closely related to ER suppression and prognosis than these other cytokines, indicating that OSM signalling may be a key feature of inflammation-based ER regulation.

Despite the clinical link between inflammation and ER suppression, the broader notion of inflammation dictating ER status is relatively novel. Beyond our studies and those referenced in the above paragraph, very little has been done to mechanistically link inflammatory pathways with ER. In fact, because IL-6 and TNF can be expressed by both leukocytes and non-hematopoietic cells such as fibroblasts, it should not be assumed that expression of these cytokines in breast cancer occurs exclusively in the context of inflammatory conditions. Although not as well studied as IL-6 or TNF, there is very little evidence to suggest that OSM is produced by non-hematopoietic cells; thus, to the best of our knowledge, OSM secretion in breast tumours is likely an inflammation-specific event. Although we are planning studies to assess this issue more directly, our preliminary gene expression analysis shown in Figure 24 supports the notion that leukocytes, most likely members of the monocyte/macrophage lineage, are the primary sources of OSM in breast tumours. It should be noted however, that neutrophils are in fact the only leukocyte type that has been directly shown to produce OSM in response to breast cancer cells (239). However, this information was derived from *in vitro* coculture analysis, and neutrophils are generally present at much lower levels than macrophages in breast tumours (362). In a rather timely publication, Stossi *et al* (324) recently demonstrated the ability of macrophages to suppress ER at magnitudes similar to those observed in our studies of OSM. Furthermore, they showed that macrophage-conditioned media caused ER loss with similar kinetics (protein-level effects observed within one day) and in a manner dependent on

MAPK signalling. They did not, however, identify the macrophage-derived factor responsible for ER suppression, making OSM a tantalizing candidate.

Our study and that of Stossi *et al* complement prior work by the El-Ashry lab that demonstrates a role for MAPK signalling in ER suppression (114, 115, 117). The assumption in these earlier studies was that hyperactive MAPK signalling occurred downstream of EGFR family receptors. However, our study of OSM clearly indicates that inflammatory cytokines can also be potent MAPK stimulants. Interestingly, although EGFR inhibition suppressed OSM-induced S100A7 expression, this was not the case with respect to ER suppression, indicating that signalling from the OSMR-gp130 complex alone is sufficient for OSM to influence ER. As described in Chapter 1, the involvement of macrophages and inflammation in tumour promotion is not a novel concept (149, 151, 154, 155, 158). However, the most common effects ascribed to these inflammatory cells have been the promotion of metastatic behaviour and angiogenesis, processes that occur in breast cancer regardless of their molecular subtype. The ability of OSM and/or macrophages to regulate ER and estrogen sensitivity thus represents a potentially important advance, as it demonstrates the potential of inflammatory factors to directly modulate a fundamental aspect of breast cancer taxonomy.

It is notable that ER suppression was necessary for the full induction of OSM-induced motility in MCF7 cells (Fig. 18). This may be related to the decreased ability of OSM to enhance STAT3 and ERK1/2 activation in the context of ER overexpression, each of which is implicated as a regulator of OSM-induced migration (235, 238, 363). ER has been shown to suppress cytokine-induced JAK2 activity through its estrogen-dependent activation of SOCS2 expression (364). In turn, JAK2 may be the principal JAK responsible for ER protein downregulation (365). Although JAK1 appears to be the major JAK utilized by OSMR, JAK2 is also involved in its signal transduction (179, 366). Intriguingly, ER knockdown or tamoxifen treatment of MCF7 cells increases the level of STAT3 phosphorylation on serine 727, a modification that is mediated by MAPK signalling and essential for full STAT3 transcriptional activity (367). Given that OSMR expression is positively regulated by STAT3 (356), ER retention may thus interfere with OSM-induced signal transduction both by preventing full engagement of JAK activity and by decreasing OSM sensitivity through the prevention of STAT3-mediated OSMR maintenance. Although early reports suggested that ER can promote invasiveness in breast cancer cells (368, 369), the current balance of evidence seems to indicate that estrogen signalling through ER has the opposite effect.

To our knowledge, we are the first to document the effect of restoring ER in the context of induced ER suppression in an ER+ cell line. Others, however, have shown that expression of ER in ER– MDA-MB-231 cells results in markedly reduced invasiveness, and that inherently migratory cells within the ER+ MCF7, T47D, and ZR75 lines exhibit reduced ER expression relative to their non-migratory neighbours (370-372). Similar observations were made by a group who reported that adhesion signalling through c-Src was necessary for ER transcriptional activity and suppression of migration (373). Overexpression of either the A or B isoform of PR in MDA-MB-231 cells has also been shown to suppress invasion (374). Other than interference with JAK-STAT signalling, a possible mechanism to explain these findings is the ability of ER to suppress expression of the NF-κB family member RelB, which is highly expressed in ER– breast cancer cells and promotes an invasive mesenchymal phenotype (375). Alternatively, ER has also been shown to promote E-cadherin expression through the suppression of slug (338). We similarly observed E-cadherin induction following estrogen stimulation of MCF7 cells (which was blocked in the context of OSM treatment (Fig. 25)). Finally, a recent report involving the study of IGF-1 biology in developing murine mammary glands revealed that ER regulates signalling from the IGF-1 receptor: prior to puberty, ER was found to interact with IRS-1 (insulin receptor substrate-1) and promote activation of the PI3K-Akt pathway, which in turn appeared to suppress Ras-ERK1/2 signalling via an inhibitory phosphorylation of c-Raf. Following puberty, however, ER levels naturally declined and, in either this setting or in $ER^{-/-}$ mice, Ras-ERK1/2 activity was elevated (376). The involvement of IRS-1 unfortunately reduces the relevance of this model to OSM signalling. Nevertheless, the fact that we observed ER-dependent suppression of ERK1/2 but maintenance of Akt activation in OSM-stimulated cells raises the interesting possibility that a similar process might take place during OSM signalling (Fig. 18). The studies described above collectively imply that ER may influence multiple signalling pathways to suppress migration, invasion and, potentially, EMT.

In support of an EMT-suppressive role for ER, circulating tumour cells (CTCs) from the peripheral blood of breast cancer patients are typically ER–, regardless of the ER status of the primary breast tumour. For example, Fehm *et al* (377) reported that among 48 patients with detectable CTCs, 85% had primary tumours that were either ER+ or PR+, but only 29% of cases had CTCs that expressed these markers. In a similar study, Gradilone *et al* (378) reported that among patients with ER+ tumours and detectable CTCs, only 38% had ER+ CTCs. None of the ER– patients had ER+ CTCs. Because CTCs are presumed to have accessed the blood stream by virtue of an invasive EMT-like process, these studies support the notion that ER restrains the migratory

potential of breast cancer cells. Further evidence of the EMT-restraining effect of ER can be found in studies that indicate the direct repression of *SNAI2* expression by ER and the repression of *SNAI1* expression by the ER target gene MTA3 (metastasis associated protein-3 (338, 379, 380)).

ER loss may also be related to stemness, based on the tendency of ER– breast tumours to express stem cell-related markers (23) and our observation that ER suppression occurs along with acquisition of CSC features. Estrogen was recently shown to suppress the stem cell-like properties of breast cancer cells based on reduced mammosphere-forming capacity and suppressed expression of *NANOG*, *POU5F1*, and *SOX2* (381). This was supported *in vivo* by Horwitz *et al* (382), who showed that T47D xenografts contain rare (≤1%) cell populations that are $CD44^{+}CK5^{+}$ and ER/PR double negative (CK5 is a marker of basal-like breast cancer cells (383)). These cells had enhanced tumourigenic capacity and, importantly, produced colonies *in vitro* and tumours *in vivo* that ultimately contained the same preponderance of ER+/PR+ cells seen in normal cultures and parent tumours. This demonstrates that a luminal breast cancer cell line (T47D) contains a small basal-like population that, by exhibiting tumourigenic capacity and the ability to generate differentiated luminal progeny, fulfills the criteria of cancer stem cells. These studies generally support the concept that ER may promote a non-motile, differentiated epithelial state in breast cancer cells, suggesting that its loss due to agents such as OSM may be a key component of metastasis. By using ER+ breast cancer cells with constitutive or inducible expression of ER, it should be possible to determine if ER loss is indeed essential for cytokine-induced metastasis and development of the associated EMT and CSC-like phenotypes.

5.1.3—Regulation of EMT- and CSC-like phenotypes by OSM

The frequently cited link between mammary EMT and CSC biology was forged in 2008 when Morel *et al* (44) and Mani *et al* (43) separately demonstrated that gene expression patterns consistent with mesenchymal differentiation tended to coincide with expression of stem cell-like features. In particular, they showed that TGFβ (at the time a well-known inducer of EMT) triggered acquisition of a $CD44^{high}CD24^{-/low}$ phenotype and promoted mammosphere formation. Mani *et al* further showed that forced expression of snail or twist resulted in similar changes and dramatically enhanced *in vivo* tumourigenesis in Ras-transformed mammary epithelial cells. Numerous exogenous stimuli appear to promote mesenchymal transitions including TGFβ, Wnt family members, ligands of receptor tyrosine kinases (and hence MAPK and PI3K signalling), Notch and integrin signalling, and

hypoxia (37, 45, 384). Several transcription factors may also play key roles in regulating EMT, including snail, slug, twist, ZEB1/2, and FOXC1/2.

EMT occurs normally during embryogenesis or in special cases in adult tissue, and is thought to occur in a perturbed manner in cancer. To distinguish between these scenarios, different forms of EMT have been proposed to fall into three possible categories (38, 384): Type 1 EMT is the process by which epithelial cells adopt mesenchymal features and migrate during embryogenesis, such as the generation of distinct germ layers during gastrulation. Type 2 EMT encompasses the transition of epithelial cells into fibroblasts in adult tissues. This normally takes place in the context of trauma and fibrosis, as occurs during wound healing. OSM has been implicated in this process in the context of renal injury, in which tubular epithelial cells of the human kidney were shown to transdifferentiate into myofibroblasts in response to OSM (385). Both of these types of EMT are tightly regulated mechanisms of tissue development and homeostasis. In contrast, Type 3 EMT represents the deregulated process that occurs during cancer progression. In this case, carcinoma cells exhibit changes reminiscent of Types 1 and 2 EMT, but do so in an irregular fashion that appears to vary significantly from tumour to tumour. Since cancer cells often fail to shift completely to a mesenchymal phenotype during Type 3 EMT and frequently retain the ability to revert back to an epithelial state, carcinoma cells are increasingly thought to enter metastable phenotypes that combine features of both epithelial and mesenchymal cells, making Type 3 EMT a rather ill-defined concept (37, 40-42). It is particularly challenging to demonstrate the existence of EMT (in the classical sense) in human tumour specimens. This is because any carcinoma cell that undergoes a full transition to a mesenchymal state would be extremely difficult to distinguish from resident fibroblasts. As such, although investigators repeatedly demonstrate that seemingly full EMTs can occur in model systems of cancer, the available evidence from intact human tumours supports at best the metastable EMT concept described above (41). Indeed, circulating tumour cells from metastatic breast and prostate cancer patients co-express epithelial and mesenchymal markers at a high frequency (386).

The major functional consequence of EMT is detachment of epithelial cells from their neighbours and acquisition of invasiveness. Thus, the loosening of intercellular adhesion via loss of E-cadherin is considered an essential hallmark of EMT (387). E-cadherin is vital to the maintenance of epithelial cell layers and polarity by serving as the core protein of epithelial adherens junctions, in which the extracellular portions of E-cadherin on one cell interact with those of its neighbours to create close adhesion complexes linking the basolateral membranes (387, 388). The intracellular

portion of E-cadherin is linked to the actin cytoskeleton by a variety of proteins, including β-catenin. Loss of E-cadherin not only promotes cell dispersal but can itself trigger EMT-like gene expression changes that promote metastatic dissemination of breast cancer cells (387, 389). This is at least partially due to the liberation of β-catenin, which can enter the nucleus following dissociation from E-cadherin to exert transcriptional effects. The data shown in Chapter 4 demonstrate that OSM stimulation causes deregulation of E-cadherin in breast cancer cells through either suppression of mRNA (and thus protein) expression or internalization of membranous protein. This is consistent with the ability of OSM to promote breast cancer cell motility and invasiveness (235, 237-239, 309, 334). We also consistently observed induction of snail and slug expression, but not other markers of EMT such as vimentin, suggesting that OSM promotes a partial EMT in a manner consistent with clinical observations (41, 386).

Although we did not demonstrate that EMT-like changes were required for this process, we also observed a clear and rapid enhancement of breast CSC features in MCF7 and T47D cells following OSM treatment, including gain of a $CD44^{high}CD24^{-/low}$ phenotype, enhanced mammosphere formation, and induction of the pluripotency genes *NANOG* and *SOX2*. The combined outcomes of incomplete EMT and gain of stem cell-like features suggest that OSM may be more aptly described as a promoter of a dedifferentiated, progenitor-like state, rather than as an inducer of mesenchymal differentiation. Indeed, Tables 4 and 5 demonstrate that in the MAQC cohort (335), *OSMR* is generally more closely correlated with CSC-associated genes than with EMT factors, particularly within ER– tumours. In this regard, it is interesting that OSM failed to induce vimentin expression in our experiments (Fig. 25), and that *OSMR* expression was unrelated to *VIM* in the ER– subset of the MAQC cohort (Table 5). Because vimentin is arguably a marker of differentiated mesenchymal cells, this supports the concept that OSM-stimulated breast cancer cells undergo dedifferentiation rather than mesenchymal transdifferentation. This is consistent with the recently reported role of OSM in promoting heart repair through dedifferentiation and gain of regenerative embryonic features in cardiomyocytes (182). OSM may also be necessary for the maintenance of various populations of bone marrow hematopoietic progenitor cells (180, 193). This situation appears to be reversed in the liver, however, in which OSM can function as a hepatocyte maturation factor (181, 217). Furthermore, OSM appears to inhibit the growth of both normal and malignant neural precursor cells (390). Like many cytokines then, the functional impact of OSM is likely dependent on the specific tissue and cellular context.

5.2—Integrating concepts from Chapters 2 to 4

Data from several studies clearly indicate that OSM has cytostatic effects on malignant cells of the breast and other organs (191, 229-232). This has led to speculation that OSM could be used as a therapy to inhibit tumour proliferation. It is now equally clear, however, that OSM potently stimulates migration and invasiveness, indicating that while therapeutic administration of OSM could potentially slow tumour growth, this advantage would likely be offset by increased tumour cell dissemination (235, 237, 238, 391). OSM-induced enhancement of migration is also seen in models of osteosarcoma and cervical carcinoma (220, 221). The data presented in this thesis support the assertion that OSM exerts a net tumour-promoting effect in breast cancer, suggesting that it should not be considered as a potential therapeutic agent, but rather as a therapeutic target.

This thesis demonstrates that OSM can exert broad effects on breast cancer cells that collectively promote an aggressive phenotype. Importantly, our experimental observations were generally very consistent with those derived from human clinical cohorts. For these analyses, it must be acknowledged that our extensive use of mRNA gene expression data was not an ideal approach to explore relationships between the OSM pathway and clinical parameters. Particularly for assessment of OSMR expression, we would have preferred to use immunohistochemistry to directly examine OSMR protein levels and distribution within intact breast tumour tissue. However, this was not an option due to a lack of validated OSMR antibodies, a situation that we are currently seeking to rectify. Nevertheless, advantages of mRNA assessment included the opportunity to utilize public microarray datasets to diversify our pool of assessable patients, and the ability to examine many genes of interest (which would have otherwise been challenging and likely cost-prohibitive using an IHC approach). A nuance of the public cohorts used in Chapters 3 and 4 is worth mentioning at this point: the mRNA expression data from the Prat cohort (23) was derived from whole tumour specimens, whereas the MAQC data (335) was derived from fine needle aspirates. The distinction is that whole sections include the complete repertoire of cell types present within a tumour, from the malignant cells to stromal components such as leukocytes. Gene expression data in this setting thus represents the pooled content of all cell populations. In contrast, fine needle aspirates are typically comprised of at least 70% neoplastic cells; this increases the odds that observed gene expression patterns are derived from malignant components, but decreases one's ability to ask questions related to stromal cells. In this respect, the Prat cohort was useful for assessment of *OSM* and leukocyte genes in Chapter 3. However, in Chapter 4 our main focus was on *OSMR* and cancer cell markers,

and we thus utilized the MAQC cohort to increase the odds that our observations were not due to gene expression from stromal cells.

Our analyses of human tumour specimens using both PCR and microarray data verified that *OSM/OSMR* levels are positively associated with *S100A7* expression, suppression of ER and ER-regulated genes, and expression of genes related to mesenchymal and stem cell differentiation. Notably, each of these features appeared to be related to clinical outcome. *S100A7*, for example, was prognostic largely in the context of *OSMR*-expressing tumours (Fig. 11) and was highly expressed in the poor prognosis group defined by high *OSMR* and PI3K signature expression (Fig. 31). Although the association of *OSMR* with poor outcome was not technically dependent on clinically determined ER status, retention of both *ESR1* and *PGR* expression nevertheless defined a good-prognosis group of *OSMR*-high patients in a small sub-analysis of the Prat cohort (Fig. 21). Finally, EMT and CSC-related genes in the ER– subset of the MAQC cohort were implicitly associated with poor response to chemotherapy by virtue of their relationship to *OSMR* (Table 5 and Fig. 30), and the association of *OSMR* with poor outcome in the Prat cohort was at least partially dependent on coexpression of a small panel of EMT/CSC-related genes (Fig. 31). It thus appears that these distinct features are unified in a clinically relevant way by their shared relationship with OSM signalling.

While S100A7, the ER regulatory network, and EMT/stemness can all be directly influenced by OSM signalling and are associated with this pathway *in vivo*, they do not appear to be regulated by identical mechanisms. S100A7 was induced by a combination of multiple pathways including STAT3, PI3K, MAPK, and perhaps even EGFR (Fig. 7, 8). Generation of a $CD44^{high}CD24^{-/low}$ phenotype was similarly dependent on both PI3K and MAPK (Fig. 28). These mechanisms are presumably also involved in mammosphere formation, given that the defined growth factors in mammosphere culture media (EGF, bFGF, and insulin) each signal predominantly through these pathways. In contrast, ER suppression appeared to be regulated exclusively by Ras-mediated MAPK signalling (Figs. 15, 16), thus implicating MAPK as the single mechanism that is shared by all three processes. Another shared feature is the requirement for OSMR expression. In our experiments we found that OSM treatment was impotent in the face of OSMR knockdown, demonstrating that signal transduction via OSMR rather than LIFR is the dominant mechanism of OSM signalling. The one possible exception was S100A7, which was still induced in the absence of OSMR, albeit at significantly lower levels. Given that OSMR knockdown by siRNA was unlikely to completely suppress OSMR expression, it is also possible that S100A7 induction is dependent on OSMR but

does not require the same strength of signal transduction as other OSMR-regulated genes. Since OSM triggers similar signalling pathways from both OSMR and LIFR (308), it is not clear why a role for LIFR was not more apparent in our studies, but this may be related to the fact that OSMR is typically expressed at higher levels than LIFR in breast cancer cells (229). Indeed, the low level of STAT3 phosphorylation induced by OSM in MCF7 cells transfected with OSMR siRNA suggests that signal transduction from LIFR alone may not reach the magnitudes required to engage certain processes (Fig. 14). This is supported *in vivo*: in Chapter 2 we were unable to detect *LIFR* mRNA by PCR in breast cancer tissues, and *LIFR* expression generally failed to significantly associate with genes of interest and clinical parameters in our analyses of microarray cohorts.

Our discussion thus far has touched upon the issue of breast cancer diversity at several points. Since we have not directly determined the global gene expression patterns elicited by OSM, it would be premature to claim that OSM signalling underlies the generation of distinct intrinsic molecular subtypes of breast cancer. However, the clear enrichment of *OSM* and *OSMR* expression in the poor prognosis Her2, basal-like, and claudin-low subtypes demonstrates that a role for OSM in the evolution of these subtypes is plausible. It is likely that breast tumours evolve based on integrated inputs from both genome-level and environmental factors. While it is unclear to what extent genomic aberrations are responsible for the generation of specific tumour phenotypes, certain common genomic patterns have emerged, an obvious example being the chromosomal gain of 17q12-q21 (the Her2 locus) that underlies the Her2 intrinsic subtype. In addition, chromosomal gains of 1q and losses of 16q appear to be frequent in low grade tumours with luminal intrinsic features. Other genomic patterns are generally more complex and difficult to assign to specific tumour phenotypes (21, 392). Although it is now conventional to conceptualize breast tumours in terms of their intrinsic subtypes, the high frequency of intratumoural heterogeneity suggests that sufficient plasticity exists within tumours to allow the adoption of multiple possible phenotypes that do not strictly adhere to any one intrinsic definition (98). As stated above, this is likely due to both genetic differences among tumour subpopulations and microenvironment influences. Notably, an early meta-analysis of intrinsic subtype classification studies demonstrated that up to a third of breast tumours cannot be definitively classified by this approach (393). Moreover, this same study demonstrated that the major distinguishing feature between different groups of intrinsic tumours was the expression of *ESR1* and related luminal genes. In terms of clinical prognosis, ER expression appears to trump intrinsic subtypes in importance. This is demonstrated most clearly by basal-like tumours, which have clinical outcomes that are indistinguishable from those of ER– non-basal

tumours (394). Even among luminal tumours with expression of ER and/or PR, the expression of ER-regulated genes is associated with favorable prognosis (395). While biologically intriguing, it is thus unsurprising that intrinsic classification has failed to supplant ER and other classical pathological parameters for use in clinical practice. These studies also support the premise that ER represents the major focal point of clinically relevant heterogeneity.

We have shown that OSM can rapidly induce a functionally ER– phenotype in breast cancer cell lines that normally display classical ER+ luminal differentiation. In addition, OSM induces features that have been previously linked with ER– breast cancer, namely, S100A7 expression and mesenchymal/CSC-like differentiation. Our observation that S100A7 is dependent on snail for expression and is highly expressed in mammospheres suggests that it could be included as a component of the EMT/CSC phenotype. Importantly, both S100A7 induction and ER loss appear to be functionally required for the pro-migratory phenotype bestowed by OSM. Coupled with our *in vivo* observations, these data collectively support the concept that breast cancer phenotypes are not definitively programmed and are at least partially dependent on the microenvironment. Perhaps most intriguingly, our studies support the contention that pressure from the immune system has the capacity to powerfully modulate breast cancer evolution and plasticity.

The relationship between aggressive ER– disease subtypes and inflammation has been recognized for some time without comprehension of its cause. Our data support the viewpoint that breast cancers can evolve to more dangerous states by exploiting sub-optimal immune responses. Beyond OSM, other immune-derived cytokines such as IL-6, IL-8, TNF, and TGFβ are also known to exert tumourigenic effects (175, 332, 333, 396). In the absence of an effective anti-tumour adaptive immune response, these cytokines can foster a smoldering self-sustained inflammatory environment, contributing to the oft-used description of cancer as a 'wound that does not heal.' For example, in addition to regulating aggressive behaviour in breast cancer cells, IL-8 and OSM are likely to promote ongoing inflammation, the former by functioning as a potent neutrophil chemoattractant and the latter by augmenting leukocyte extravasation via endothelial activation (198, 199). This raises a possibility described in Chapter 1, namely, the potential for a new model of breast cancer evolution based on the quality and magnitude of the anti-tumour immune response: it is clear that good prognosis breast tumours with relatively benign histopathology (e.g. low grade and ER expression) have a limited degree of inflammation, while tumours with high risk features (e.g. high grade and ER– status) are typically considerably more inflamed (124, 125, 168, 170). However, in recent years it has also become clear that subsets of these commonly inflamed and supposedly

aggressive tumours have a favourable prognosis near par with that of non-inflamed, well differentiated lesions—these are characterized by the presence of a robust adaptive immune response featuring high numbers of T and B cells (130-132, 170). One could thus argue that the only breast cancers with a truly poor prognosis are those that harbour unproductive inflammatory infiltrates. However, cancer cells that are highly sensitive to cytokines could form a kink in this idea. Tumours containing such cells may be able to exploit and withstand all but the most herculean immune attacks. The data shown in Figure 33 support this possibility by demonstrating that ER– tumours with high *OSMR* expression appear to have a dismal prognosis regardless of the presence of robust T cell infiltrates. For the cancer immunologist, this should indicate that tackling breast cancer's ability to exploit immune responses may be an essential component of any successful immunotherapeutic strategy.

5.3—Future directions

5.3.1—Molecular aspects of OSM action

Numerous questions, both broad and precise in scope, remain open for investigation with respect to the roles of OSM in breast cancer. Many of the molecular mechanisms underpinning our observations are intriguing and, at this point, uncharacterized. In the case of S100A7, it is still not entirely clear how OSM triggers its expression. As noted in Chapter 2, the delayed kinetics of S100A7 induction following OSM exposure imply the involvement of one or more intermediary factors that are directly regulated by OSM signalling and influence S100A7 in turn. A possible candidate is snail, given that its expression is induced by OSM and is necessary for S100A7 expression. It is even less clear how S100A7 mRNA and protein remains stably expressed for several days following withdrawal of OSM exposure. Although we determined a role for S100A7 in mediating OSM-induced migration, we did not assess its other possible effects. Particularly intriguing is the possibility that S100A7 is secreted and exerts functions via activation of pattern recognition receptors such as RAGE and TLR4. In this manner, OSM could indirectly regulate inflammatory responses by eliciting production of immunomodulatory factors from neoplastic cells. Work is currently underway in our lab to generate recombinant S100A7 expressed from human cells that can be used for exploration of this last question.

The mechanism by which OSM causes ER suppression remains murky. Although we have shown MAPK signalling to be involved, we were unable to do more than partially block ER suppression using MAPK pathway inhibitors, implying that a separate pathway is also involved.

However, none of the other signalling pathways that we tested appeared to be individually required for ER suppression, including PI3K and NF-κB, which have both been previously implicated as ER-suppressive factors (116, 119, 397). Combinatorial blockade of multiple pathways did not resolve this issue (for example, kinase inhibitor treatment in addition to STAT3 siRNA (data not shown)). Exactly how MAPK signalling blocks ER expression in this context is also unclear, but may involve direct activity of ERK2 at the *ESR1* promoter (324).

Further investigation of OSM-induced EMT/CSC features is warranted. For example, is S100A7 truly a marker of EMT and, if so, what is its specific role in this context? Given that both MCF7 and T47D cells appeared to undergo an OSM-induced EMT-like process, but with few shared changes in gene expression (e.g. snail and slug induction), what are the minimal requirements for OSM-induced EMT? A very intriguing but unexplained observation was that not all tumour cells respond equally to OSM, even though in 2-dimensional culture they should have roughly equal cytokine exposure. This is most clearly evident by microscopy. As shown in Figure 25, many cells retain epithelial morphology and intercellular adherens junctions, while others (to varying extents) break away from their neighbours to shed their epithelial polarity and migrate. What explains this heterogeneity? One possibility is variance in receptor expression: the flow cytometry data shown in Figure 28 demonstrates that the baseline range of OSMR expression within both MCF7 and T47D populations may span up to two logs. An additional possibility is that the pro-migratory effects of OSM compete with the pro-epithelial influence of intercellular adhesion. That is, cells at the periphery of a cluster have fewer contacts with their neighbours than those at the centre, and could thus be more proficient at dismantling their remaining adhesion structures to become independent.

In terms of stem cell biology, we have established that OSM induces a CSC-like phenotype using the two most common methods of *in vitro* characterization: profiling for $CD44^{hi}CD24^{lo/-}$ status and propagation of single cells as mammospheres. Our assessment of the pluripotency genes Oct4, Nanog, and SOX2 (and identification of SOX2 as a factor of interest) was actually a somewhat unusual strategy for a study of this type. A common assay that we did not exploit was the assessment of 'side-populations,' cells that rapidly efflux the membrane-permeable DNA-binding dye Hoechst 33342 by virtue of stem cell-associated transmembrane drug pumps (49). These cells can be identified and isolated via their distinct low-fluorescence pattern during FACS analysis. However, Hoechst 33342 is excitable by UV wavelengths, and we were unable to assess side populations due to the absence of an appropriate laser in our instruments. Ultimately, the most important question that we did not address was whether OSM can influence tumourigenicity in immune deficient mice.

5.3.2—Exploration of OSM signalling in animal models

Planning is currently underway in our lab for experiments on OSM function in animal models, which will jointly support investigations of EMT, stemness, and ER suppression. The simple question of whether OSM promotes tumourigenicity (the standard *in vivo* CSC assay) can be addressed by pretreating cells with OSM *in vitro*, injecting them at varying titers into mice, and observing the frequency of tumour outgrowth. For more complex questions involving processes within developing and established tumours, the most straightforward model would be to engineer breast cancer cells to inducibly express OSM. A tetracycline-inducible system is preferred because this would allow temporal control of OSM expression (that is, one could establish a tumour in nude mice and then initiate OSM expression to, for example, determine ER expression or metastatic dissemination after defined periods of time). This model is limited, however, in that it does not recapitulate the inflammatory environment in which OSM is thought to function. This problem is particularly pertinent given our observations that OSM can exert synergistic effects with other inflammatory cytokines such as TNF (indeed, exploration of the effects of different cytokine combinations is another goal for future study). We are thus considering a more complex strategy involving intravenous or intratumoural delivery of human monocyte-derived macrophages engineered to express OSM (398, 399). These would be expected to produce multiple cytokines other than OSM upon tumour infiltration, thereby providing a more realistic approximation of the cytokine environment during inflammation. To determine the specific effects of OSM in this setting we would perform parallel experiments involving xenografts of tumour cells with abrogated OSMR expression (e.g. via tetracyline-inducible shRNA). Key questions to address include whether OSM can suppress ER and ER-dependent functions *in vivo*, whether OSM influences the response of breast tumours to endocrine or chemotherapy, and, relevant to the concepts of EMT and CSCs, whether OSM enhances tumour dissemination and metastasis.

To the best of our knowledge, the role of OSM in breast cancer has never been investigated using animal models, making it difficult to predict the outcomes of the studies proposed above. For example, the cytostatic effect exerted by OSM on conventionally cultured breast cancer cells suggests that OSM may inhibit primary tumour outgrowth, whereas the mammosphere-promoting effect of OSM suggests otherwise. In addition, if the major effect of OSM is to make breast cancer cells more motile and capable of surviving exacting conditions, we may see a greater effect of OSM on the metastasis of implanted tumours than on growth of the primary mass. In terms of endocrine therapy, will the ER-suppressive effect of OSM enhance the ability of endocrine-disrupting agents to

inhibit tumour growth, or will OSM-stimulated cells display adaptations that render them resistant to endocrine therapy? It should be noted that human xenograft models are required for addressing questions involving ER and endocrine therapy because murine breast cancers rarely express ER (288), and little or no data currently exist regarding the effects of endocrine therapy in syngeneic models of murine cancer. As a result, there are no well characterized ER+ murine breast cancer cell lines, and our current knowledge of the effects of ER biology in preclinical breast cancer models is derived primarily from the study of immune compromised mice. This represents an interesting (if vexing) example of how similar diseases do not necessarily follow the same pathways of molecular evolution in mice and men.

Our analysis of the MAQC cohort provided evidence that OSM signalling may be related to chemotherapy resistance. Similarly, because many of the cases in the Prat cohort (particularly those negative for ER) would have also received chemotherapy, the relationship between *OSMR* expression and poor prognosis also supports this concept. This should be experimentally verified in future studies. Because chemosensitivity is a distinct issue from that of endocrine sensitivity, it is helpful that immunocompetent mice and syngeneic tumours could be used for such investigation. This is particularly pertinent given our growing appreciation that the host immune system is critical for the efficacy of several cytotoxic therapies, including the widely deployed anthracycline drug family (144, 146, 400, 401). Thus, reliably addressing questions regarding the effects of chemotherapy may require the use of immunocompetent animal models.

5.3.3—Assessment of OSM signalling in the clinical setting

Our analyses of human cohorts provide confidence that our *in vitro* results will be reproducible in animal models. Since most cases of breast cancer recurrence and mortality are due to distant metastasis, the apparently strong association of OSMR with poor prognosis supports a role for OSM signalling in tumour dissemination, outgrowth of metastatic tumours, or both. Further exploration of human cohorts is required, however. Detailed characterization of the cellular distribution of OSMR expression in whole breast tumour sections (currently lacking due to the absence of well-characterized OSMR antibodies for IHC) will be essential. In addition to permitting more rigorous clinical study of OSMR, this will improve our ability to interpret data derived from mRNA-profiling studies. Importantly, at least two distinct isoforms of OSMR exist that are generated by alternative mRNA splicing: the full length form and one or more truncated, soluble forms that may act as decoy receptors (402, 403). This does not affect our interpretation of the data generated from the MAQC

cohort, as every *OSMR* probe included in that microarray platform recognizes only the full length *OSMR* isoform. However, a small fraction of the probes from the microarray platform used in the Prat cohort recognize the alternative *OSMR* isoform(s), such that roughly 10% of the *OSMR* probe signal is likely due to transcript variants. If OSMR variants are expressed in breast cancer and exert distinct biological functions, IHC studies involving antibodies that can distinguish between these isoforms will thus be critical for proper comprehension of the clinical relevance of OSMR expression. The influence of OSM signalling on ER makes a strong case for determining its clinical contribution to endocrine therapy resistance. Interrogation of OSM and OSMR expression in uniformly endocrine-treated patient cohorts with complete clinical history and follow-up data will be necessary to address this issue. Similar analyses should be performed with respect to chemotherapy outcome in both ER– and ER+ patients.

5.3.4—Impact of OSM signalling on the evolution of breast cancer molecular subtypes

When attempting to publish a version of the manuscript associated with Chapter 3, we encountered vehement opposition from a reviewer who (incorrectly) believed we were claiming that OSM could cause luminal breast cancers to evolve into other molecular subtypes such as basal-like or claudin-low tumours. This is not a point we argued in that study, since we did not have the appropriate global gene expression data required to make that claim. However, our data did suggest that OSM-treated cells lost key features of luminal differentiation (ER and PR) and that *OSM/OSMR* expression was strongly associated with basal-like and claudin-low tumours *in vivo*. Although the reviewer seemed to believe this, we are unaware of any compelling evidence to indicate that such evolution from one molecular subtype to another is impossible. In fact, Haughian *et al* (404) recently supported this concept by demonstrating that ER+/PR+ luminal breast cancer cell lines contain hormone receptor negative subpopulations that are selectively propagated in response to endocrine therapy or estrogen withdrawal. Importantly, these hormone-independent subpopulations had gene expression signatures consistent with basal-like and claudin-low disease. It would thus be fascinating to generate gene expression array profiles for OSM-treated breast cancer cells. In this manner, one could determine if OSM-treated luminal cells (such as MCF7 and T47D) do indeed acquire a gene expression signature indicative of basal-like or claudin-low differentiation. One could profile tumours in animal models in a similar fashion. For example, the commonly used MMTV-Her2 transgenic mouse produces (somewhat counter-intuitively) mammary tumours with gene expression patterns akin to luminal disease (288); it would be interesting to determine if OSM can substantially

alter this phenotype. Demonstrating the ability of OSM to convert breast tumours from one identifiable molecular subtype to another would have profound implications for the role of the microenvironment in governing breast cancer evolution.

5.3.5—What are the cellular sources of OSM in breast cancer?

The specific cell populations that generate OSM in breast tumours are currently unknown. While we have seen no evidence of OSM expression in breast cancer cells, and OSM expression correlates strongly with myeloid leukocyte infiltration in breast tumours, direct cellular analysis is required to conclusively resolve this issue. To this end, we are planning studies in which prospectively accrued samples of breast tumours will be processed to provide matching fresh, FFPE, and frozen specimens; the fresh samples will be disaggregated and analyzed by FACS using lineage markers to identify distinct cell types, which will then be isolated and profiled by Q-PCR for expression of *OSM* and other genes of interest. The preserved specimens will be deployed in various assays to confirm FACS-based results (for example, laser capture microdissection of frozen samples to isolate and analyze various stromal and epithelial populations, or ELISA-based quantification of OSM levels in disaggregated tumour specimens). We anticipate that this study will provide a much more precise identification of the range of OSM-producing cell types in breast cancer.

5.3.6—OSM signalling as a therapeutic target

If the studies described above support a role for OSM signalling in breast cancer progression, the OSM pathway may be an attractive target for the development of novel therapies. The identification of the cellular source of OSM will allow us to determine if depletion or inhibition of a particular culprit is feasible. For example, if tumour-associated macrophages are a dominant source of OSM, these could be selectively depleted with agents such as bisphosphonate-loaded liposomes (405). Alternatively, if T cells are implicated, T cell inhibitory agents including alefacept and efalizumab are now clinically approved and in use for conditions such as psoriasis (406). If one is interested in therapeutically augmenting T cell-mediated immunity, however, inhibition of T cells may obviously not be an ideal strategy.

Receptor-ligand interactions could be impaired in several ways, including through the use of antibodies designed to either neutralize OSM or block access to OSMR. The latter method would have to be approached with caution, however, as many cell types express OSMR (particularly endothelial cells). This means that complete antibodies with the potential to elicit cell- or

complement-mediated cytotoxicity may have dangerous off-target effects. An Fab fragment, which retains antigen binding capacity but lacks immune-stimulatory activity, might be safer. The soluble form of OSMR could also be exploited clinically as an alternative OSM-neutralizing agent. Antibody and recombinant receptor based therapies that target inflammatory cytokines such as IL-6 (e.g. toclizumab) and TNF (e.g. etanercept) are now established, and provide a helpful precedent by showing that therapeutic blockade of cytokine signalling is clinically tractable (330, 407). While specific inhibition of OSMR signalling could be achieved using rationally designed small molecules, this strategy is complicated by the lack of a targetable catalytic site in the OSMR/gp130 complex (recall that they signal via janus kinases). JAKs, however, function via kinase domains that are readily targetable by small molecules, and JAK inhibitors are currently under clinical testing with promising evidence of efficacy in diseases such as rheumatoid arthritis (408). A disadvantage of JAK inhibition is the generally broad suppression of immune responses, given that most immunomodulatory cytokines operate through JAK signalling. Thus, the ability to target OSMR specifically would be ideal. The successful design of small molecules that inhibit the interaction of IL-2 with its cognate receptor (IL-2Rα) provide reason for optimism that a similar feat is achievable with respect to OSM (409, 410). Several key amino acid residues of human OSM are crucial mediators of receptor interaction and may thus provide useful targets for rational drug design: residues critical for gp130 recognition include Gln16, Gln20, Gly120, and Asn124; those necessary for interaction with OSMR and LIFR include Phe160 and Lys163 (411). The mild phenotypes of OSM and OSMR knockout mice imply that a specific anti-OSM/OSMR therapy would have relatively limited side effects (180, 216). Nevertheless, the documented roles of OSM in maintenance of liver and heart tissue integrity suggest that caution should prevail (181, 182). For breast cancer patients, the ability of OSM to promote cardiac repair is particularly relevant, given that cardiac toxicity is a major limitation of widely used breast cancer therapies (with which OSM inhibition could be feasibly combined) such as trastuzumab and anthracyclines.

Among the potential benefits of targeting OSM, the restoration of ER expression and ER-driven biology is prominent. Breast tumours with restored ER expression may be targetable with endocrine therapy (117) and may acquire increased luminal differentiation, with consequent inhibition of the pro-metastatic EMT/CSC phenotype. Blockade of OSM signalling could also impair pro-metastatic behaviour that is independent of ER. In addition to endocrine therapy, this approach may render breast tumours more sensitive to chemotherapy, other targeted agents such as EGFR-family inhibitors and, potentially, immunotherapy.

5.4—Conclusions

OSM first garnered interest as a potentially therapeutic cytokine due its broad cytostatic effects on cancer cells. In the field of breast cancer, our work adds to a growing body of literature that supports an opposite perspective. While OSM does indeed slow the proliferation rate of breast cancer cells, a reproducible trade-off is an increased propensity for migration and invasion. We have expanded on these observations by demonstrating that, in what are otherwise well differentiated ER+ breast cancer cells, OSM causes concurrent suppression of ER and ER-regulated functions, gain of the oncogenic factor S100A7, phenotypic changes consistent with a mesenchymal transition, and gain of features associated with breast cancer stem cells. This complex phenotypic shift occurs rapidly, within no more than two days. Importantly, the above OSM-induced features are known to be frequently coincident in aggressive, poor prognosis breast cancers. Indeed, OSM and OSMR expression in human tumours was reproducibly associated with each of these features, in addition to poor prognosis. It therefore appears that OSM signalling is a viable explanation for why ER-negativity, S100A7 expression, mesenchymal/stem cell differentiation, and inflammation tend to occur simultaneously in breast cancer. Tissue microenvironments have long been known to fundamentally regulate basic biological processes such as embryonic cell differentation and morphogenesis. OSM thus adds further weight to the growing recognition that microenvironment processes are equally important in cancer pathogenesis.

Because OSM is presumed to be a product of intratumoural leukocytes, it is fitting to conclude our discussion of OSM by exploring a hypothetical (and probably naïvely simplistic) model of its role during tumour inflammation (displayed graphically in Figure 34). As tumours develop and grow, malignant cells at the core of a growing mass are inevitably isolated from the local blood supply, depending on the tumour's ability to induce local angiogenesis. Many of these oxygen and nutrient-deprived cells ultimately die by necrosis, which liberates intracellular constituents that alert resident hematopoietic cells to the presence of tissue trauma. These include factors such as HMGB1 (high mobility group box-1) and HSPs (heat shock proteins) that are collectively known as damage-associated molecular patterns (DAMPs). Local tissue-resident leukocytes (largely macrophages and dendritic cells) are stimulated by these tumour-derived DAMPs to produce soluble inflammatory mediators such as the early pro-inflammatory cytokine IL-1, which collectively encourage the activation (and increased permeability) of local endothelial cells and influx of additional leukocytes. These will initially include innate immune cells such as neutrophils and monocytes (which differentiate in the tissue to become macrophages), but at later stages will involve increasing

numbers of adaptive lymphocytes (T and B cells). It is unclear at what stage of tumour inflammation OSM is produced but, given its shared biology with other inflammatory cytokines and the known ability of macrophages and dendritic cells to produce it, OSM may be relevant even at this early point in the inflammatory cascade. What happens from here on is murky, given the wide spectrum of inflammatory states observed in breast cancer. However, if we assume that the tumour persists in the face of mounting inflammation, which will include elements of both innate and adaptive immunity, we can also assume that OSM will be an increasingly prominent component of the cytokine milieu.

Provided that they express sufficient levels of OSMR, tumour cells in close proximity to OSM-secreting leukocytes could respond to OSM in several possible ways. ER may be suppressed, leading to insensitivity to estrogen and probable resistance to endocrine therapy. Loss of other epithelial features that are regulated by ER may accompany its loss, including hallmark luminal differentiation genes such as PR and GATA3. These cells may also downregulate cell adhesion complexes (particularly given the ability of ER to support E-cadherin expression), upregulate promigratory machinery (including S100A7), and invade surrounding tissues to access the circulatory system. In addition, OSM stimulated cells may enhance expression of stem cell-like features, with the potential consequence of acquired resistance to chemotherapy. These migratory cells may express genes reminiscent of mesenchymal differentiation, including expression of key transcription factors such as snail and slug. The combined effects of enhanced tumour cell migration and increased blood vessel permeability (both of which can be mediated by OSM) are likely to collectively promote the intravasation and dissemination of cancer cells. This may explain why cancers of the frequently inflamed basal-like subtype appear to favour a hematogenous route of dissemination (412). Ultimately, the cancer stem cell theory suggests that the cells most likely to survive at distant sites and produce clinically relevant metastases are those with a stem-like phenotype, by virtue of their inherent resistance to apoptotic stimuli and their enhanced ability to generate new tumours.

The combined effects of OSM may therefore underlie several necessary steps of breast cancer metastasis. OSM may also promote therapeutic resistance, and could be a significant factor in the generation of breast cancer heterogeneity and the etiology of aggressive disease subtypes. Through production of pleiotropic cytokines such as OSM and the failure to eradicate neoplastic cells, a suboptimal anti-tumour immune response thus renders itself susceptible to exploitation and, in many cases, likely plays a critical role in supporting pathogenesis.

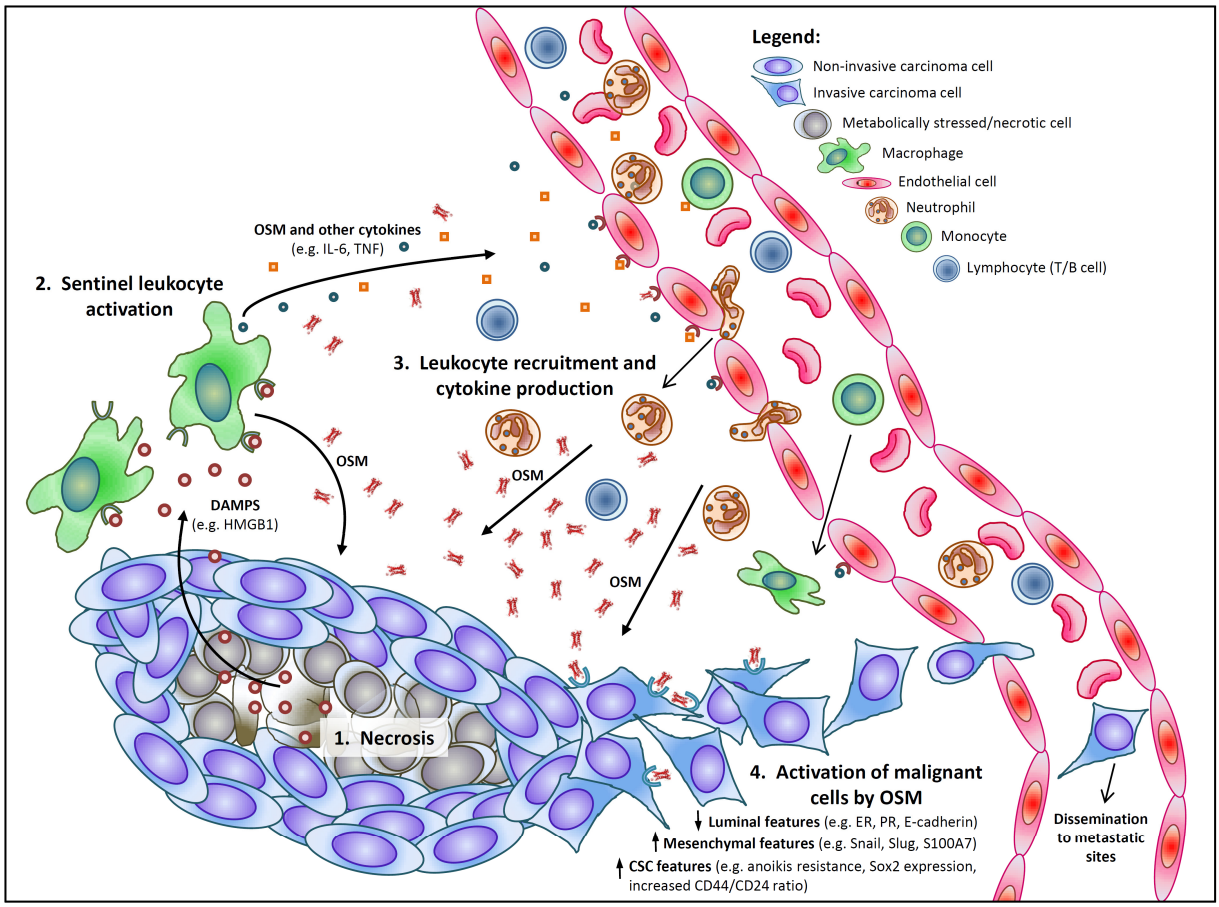


Figure 34. **Hypothetical model of OSM effects during tumour inflammation**. Cancer cells at the core of the growing tumour become increasingly excluded from vasculature, undergo necrosis due to lack of oxygen and nutrients, and release damage-associated molecular patterns (DAMPS (**1**)). DAMPS activate tissue-resident leukocytes (**2**), causing them to release pro-inflammatory cytokines that promote endothelial cell activation and leukocyte recruitment (**3**). OSM produced by infiltrating leukocytes induces phenotypic changes in malignant cells (**4**), including reduced epithelial differentiation and gain of metastatic abilities.

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APPENDICES

Appendix A. Primers and PCR cycling parameters from Chapter 2

Target	Primer 1 (5' to 3')	Primer 2 (5' to 3')
S100A7	AAGAAAGATGAGCAACAC	CCAGCAAGGACAGAAACT
GAPDH	ACCCACTCCTCCACCTTTG	CTCTTGTGCTCTTGCTGGG
CD130	TCTTCTGGGAGTGCTGTTCTGCTT	AGGTGACCACTGGGCAATATGACT
OSMR	GTTTGCTTGGCTGGGCTAC	TGGTAAGTCCTCAAGGACAGC
IL6R	CTCCTGCCAGTTAGCAGTCC	TCTTGCCAGGTGACACTGAG
OSM	GGGGGTACTGCTCACACAGA	CTGTCTGCTTCTGGAGCTGG
IL6	AGTGAGGAACAAGCCAGAGC	CATTTGTGGTTGGGTCAGG
LIF	TGAAGTGCAGCCCATAATGA	TGAGGTTGTTGTGACATGGG

PCR reactions were performed in 20 µl volumes with the following components: 20 mM Tris-HCl (pH 8.4), 50mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 2 µM forward and reverse primers, and 5 U/µl *Taq* DNA polymerase. One µl of each cDNA sample was amplified as follows: 5 min at 95°C, followed by 35 cycles of 45 sec at 95°C, 30 sec at 60°C, and 30 sec at 72°C. Amplification of S100A7 required only 30, and ERα and GAPDH 25 cycles with an annealing temperature of 55°C.

Appendix B. Q-PCR primers from Chapter 4*

Target	Primer 1 (5' to 3')	Primer 2 (5' to 3')
OSM	CTCGAAAGAGTACCGCGTG	TCAGTTTAGGAACATCCAGGC
OSMR	TCCCAATACCACAAGCACAG	GCAAGTTCCTGAGAGTATCCTG
S100A7	CCCAACTTCCTTAGTGCCTG	CTCTGCTTGTGGTAGTCTGTG
CDH1	CCCAATACATCTCCCTTCACAG	CCACCTCTAAGGCCATCTTTG
CDH2	CCCAAGACAAAGAGACCCAG	GCCACTGTGCTTACTGAATTG
SNAI1	GGAAGCCTAACTACAGCGAG	CAGAGTCCCAGATGAGCATTG
SNAI2	ACACATTAGAACTCACACGGG	TGGAGAAGGTTTTGGAGCAG
TWIST1	CTCAGCTACGCCTTCTCG	ACTGTCCATTTTCTCCTTCTCTG
FOXC1	AGTAGCTGTCAAATGGCCTTC	TGCCTTGATGGGTTCCTTTAG
ZEB1	ACCCTTGAAAGTGATCCAGC	CATTCCATTTTCTGTCTTCCGC
VIM	CGTGAATACCAAGACCTGCTC	GGAAAAGTTTGGAAGAGGCAG
FN1	ACTGTACATGCTTCGGTCAG	AGTCTCTGAATCCTGGCATTG
POU5F1	AGAACATGTGTAAGCTGCGG	GTTGCCTCTCACTCGGTTC
NANOG	GAAATACCTCAGCCTCCAGC	GCGTCACACCATTGCTATTC
SOX2	CACACTGCCCCTCTCAC	TCCATGCTGTTTCTTACTCTCC
RPL27	CAATCACCTAATGCCCACAAG	TTCTTGCCTGTCTTGTATCTCTC

*All used at 60°C annealing temperature

Appendix C. Recurrent abbreviations and acronyms

BCS	breast conserving surgery
C/EBP	C/CAAT enhancer binding protein
CSC	cancer stem cell
CTC	circulating tumour cell
DCIS	ductal carcinoma *in situ*
DFS	disease-free survival
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial-to-mesenchymal transition
ER	estrogen receptor-alpha
ERβ	estrogen receptor-beta
ERK	extracellular regulated kinase
Estrogen/E2	17-beta-estradiol
Her2	human epidermal growth factor receptor-2
HR	hazard ratio
IFNγ	interferon-gamma
IGF-1R	insulin-like growth factor-1 receptor
IHC	immunohistochemistry
IL	interleukin
IL6R	interleukin-6 receptor
JAK	janus kinase
LBA	ligand-binding assay
LIF	leukemia inhibitory factor
LIFR	leukemia inhibitory factor receptor
MAPK	mitogen-activated protein kinase
MBTB	Manitoba Breast Tumour Bank
MFI	mean fluorescence intensity
NF-κB	nuclear factor kappa-B
OR	odds ratio
OSM	oncostatin-M
OSMR	oncostatin-M receptor-beta
PBS	phosphate-buffered saline
pCR	pathological complete response
PI3K	phosphatidylinositol-3-kinase
PR	progesterone receptor
Q-PCR	quantitative polymerase chain reaction
ROC	receiver-operator characteristic
r_s	Spearman's correlation coefficient
RT-PCR	reverse-transcription polymerase chain reaction
SD	standard deviation
SEM	standard error of the mean
STAT	signal transducer and activator of transcription
TAM	tumour-associated macrophage
TDLU	terminal duct lobular unit
TGFβ	transforming growth factor-beta

TIL	tumour-infiltrating leukocytes
TNBC	triple negative breast cancer
TNF	tumour necrosis factor-alpha
TNNB	triple-negative, non-basal
Treg	regulatory T cell
UNC	University of North Carolina
VEGF	vascular endothelial growth factor